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Modulation of invariant NKT cell activity by cytokines and receptors in human disease

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To my family

"Det löser sej"
Timbuktu, 2005

ABSTRACT

Invariant natural killer T (NKT) cells are innate-like lymphocytes with both immunoregulatory and cytotoxic functions that play a role as activators and regulators of the immune response in many diseases. NKT cells are believed to bridge the innate and adaptive immune systems by rapidly producing large quantities of cytokines after recognition of CD1d-presented lipid antigens. NKT cells can be divided into two phenotypically and functionally distinct subsets based on the expression of CD4. How the NKT cells and their subsets are regulated, and how they integrate signals from their environment to modulate immune responses is still not fully understood.

In this thesis I have investigated how the activity of NKT cells can be modulated by factors other than the TCR, such as cell surface receptors and cytokines. These investigations have been based on blood samples from healthy controls, and from patients suffering from HIV-1 infection or atopic eczema (AE). We have found that CD4- NKT cells are able to degranulate and kill target cells in an NKG2D-dependent but TCR-independent manner in response to NKG2D stimulus. Moreover, we have shown that NKG2D+ NKT cells frequently express perforin that polarizes toward NKG2D-ligand expressing tumor cells. These data demonstrate that the CD4- subset of human NKT cells can mediate direct lysis of CD1d-negative target cells upon NKG2D engagement. We have further characterized the phenotype and function of NKT cells in patients with chronic diseases. In patients with chronic HIV-1 infection, the CD4- NKT cell subset showed increased expression of the inhibitory programmed death-1 (PD-1) receptor, and displayed severe functional defects. However, the functional impairment was not caused by PD-1 expression *per se* because the defect could not be reversed by PD-1 blockade. In addition, we have studied the effect of interleukin-2 (IL-2) on NKT cells and natural killer (NK) cells in patients with chronic HIV-1 infection. Material for this study was obtained from a longitudinally study, where administration of IL-2 was added to the antiretroviral treatment (ART) for one year. We found that NKT cells and NK cells responded with different kinetics and in different ways to the IL-2 administration. The NKT cells responded with a gradual numerical increase, but with no significant functional changes. NK cells responded rapidly with an expansion of the cytotoxic $CD56^{dim}$ NK cell subset and increased IFN-γ production. However, the effects of IL-2 on these cells were generally not sustained post treatment. NKT cells were also studied in the chronic inflammatory skin disease AE where the patients have elevated levels of plasma IL-18. Our data provide evidence that IL-18 is a potent activator of human NKT cells promoting an acute pro-inflammatory CD1d-dependent response, even in the absence of exogenous lipid antigens. Interestingly, chronic exposure of NKT cells to IL-18 is inhibitory and skews the NKT cell pool by selectively suppressing the proliferation of CD4+ NKT cells. Importantly, our *in vitro* data are reflected in AE patients where reduced numbers of CD4+ NKT cells are associated with elevated levels of IL-18 and disease severity.

In conclusion, the work presented here contributes to our understanding of the function and role of NKT cells in human diseases including infections and allergies.

LIST OF PUBLICATIONS

This thesis is based on two publications and two manuscripts. The individual papers are referred to by roman numerals.

I. **Kuylenstierna C**, Björkström NK, Andersson SK, Bosnjak L, Malmberg KJ, Ljunggren HG, Moll M and Sandberg JK. NKG2D-mediated triggering of TCR-independent NK cell-like cytolytic activity in human CD4-negative CD1d-restricted NKT cells. 2010. S*ubmitted.*

II. Moll M, **Kuylenstierna C**, Gonzalez VD, Andersson SK, Bosnjak L, Sonnerborg A, Quigley MF and Sandberg JK. CD1d-restricted NKT cells retained in chronic HIV-1 infection exhibit decreased function and elevated PD-1 expression. Eur. J. Immunol. 2009 Mar;39(3):902-11.

III. **Kuylenstierna C**, Snyder-Cappione JE, Loo CP, Long BR, Gonzalez VD, Michaelsson JM, Moll M, Spotts G, Hecht FM, Nixon DF, and Sandberg JK. NK cells and CD1d-restricted NKT cells respond in different ways with divergent kinetics to IL-2 treatment in primary HIV-1 infection. 2010. *Submitted.*

IV. Lind S, **Kuylenstierna C**, Moll M, Domange-Jordö E, Winquist O, Lundeberg L, Karlsson M, Tengvall-Linder M, Johansson C, Scheynius A, Sandberg JK, and Karlsson MC. I. IL-18 skews the invariant natural killer T cell population via autoreactive activation in atopic eczema. Eur. J. Immunol. 2009 Aug;39(8):2293-301.

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LIST OF ABBREVIATIONS

α-GalCer	Alpha-galactosylceramide
ADCC	Antibody-dependent cellular cytotoxicity
AE	Atopic eczema
AHR	Airway hyperreactivity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral therapy
AZT	Azidothymidine
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytolytic T lymphocytes
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3 grabbing non-integrin
DNAM-1	DNAX adaptor molecule 1
FACS	Fluorescence-activated cell sorting
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HSV1	Herpes simplex virus type 1
IFN	Interferon
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
KIR	Killer cell immunoglobulin-like receptor
LCMV	Lymphocytic choriomeningitis virus
LFA-3	Leukocyte function-associated antigen-3
LPS	Lipopolysaccaride
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MIC	MHC class I chain-related
MIP	Macrophage inflammatory protein
Mtb	Mycobacterium tuberculosis

MTOC	Microtubule-organizing center
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NKG2D	NK group 2 member D
NKR	NK cell receptor
NKT cell	Natural killer T cell
PD-1	Programmed death-1
pDC	Plasmacytoid dendritic cell
SAP	SLAM-associated protein
SIV	Simian immunodeficiency virus
SLAM	Signaling lymphocytic activation molecule
T1D	Type 1 diabetes
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis-inducer
Tregs	Regulatory T cells
ULBP	UL16 binding protein
WHO	World health organization

1 INTRODUCTION

The immune system is there to protect the host from invading pathogens, such as viruses and bacteria. By studying immunology we wish to gain better understanding of the means by which the immune system controls infections. A deeper understanding can hopefully also contribute to new ways to improve vaccination and treatment strategies against infections, and new ways to alleviate deficiencies in the immune system.

The immune system is composed of many different cell types with distinct functions and distributions in the body, as well as soluble molecules, such as cytokines and antibodies. In vertebrates, the immune system can be divided into two main branches, the innate and the adaptive immune system. This division is not always clear-cut, but can still help us think about principal differences between the various immune defense mechanisms. The innate immune system is the first line of defense and acts rapidly and with generally encoded fixed specificities. In contrast, adaptive immune responses take days to develop and display exquisite antigen specificity against the invading pathogen. The adaptive responses also establish an antigen-specific immunological memory after the infection is cleared. The cells in the innate arm of the immune system include e.g. dendritic cells (DCs), granulocytes, monocytes/macrophages and natural killer (NK) cells, whereas T cells and B cells are generally viewed as cells of the adaptive immune system.

This thesis is focused primarily on natural killer T (NKT) cells, which are cells bridging the innate and adaptive immune systems. NKT cells are unconventional T cells expressing an invariant T cell receptor (TCR), but they have innate characteristics in that they are activated early in infection and rapidly secrete cytokines upon activation. With these competences, NKT cells have the possibility to influence many cells, both innate and adaptive, towards different directions early in the immune response. They are generally regarded as immunoregulatory cells and could therefore be potential targets for therapeutic intervention.

1.1 THE INNATE AND THE ADAPTIVE IMMUNE SYSTEM

The innate immune system is found in all living organisms from birth and is genetically programmed to detect general structures of foreign invading microbes. The innate immune system is composed of specialized cells, soluble molecules, as well as physical barriers, such as the epithelial cell layers in the skin and the mucus layers in the respiratory tract. Many innate immune cells express pattern-recognition receptors (PRRs) that recognize common molecule patterns expressed on the surface of many microbes, so called pathogen-associated molecular patterns (PAMPs). Pathogens express different PAMPs, to which the immune system responds in different ways. A well-known family of PRR is the Toll-like receptor (TLR) family that is involved in many innate reactions. For example, TLR-4 recognizes lipopolysaccaride (LPS) on gram-negative bacteria and TLR-3 recognizes viral double-stranded RNA. The innate cells also recognize other cellular stress signals that indicate infection or transformation. NK cells are involved in the detection of such signals and this will be discussed into more detail later. The complement system, another innate arm, is composed of soluble plasma proteins that are activated by antigen-antibody complexes or microbial surfaces. Through a cascade of interactions protease complexes are formed that make cells more susceptible to phagocytosis, as well as induce a series of inflammatory responses to help fight infection. Activation of innate cells by any of the different signals or molecules mentioned above, leads to killing through phagocytosis or direct cytotoxicity and/or activation of the adaptive immune response by cytokines and co-stimulatory molecules [1-3].

The adaptive immune system is not fully mature at birth, but develops and changes throughout life when the organism is exposed to different antigens. In contrast to the innate immune system, the adaptive immune system forms immunological memory directed against encountered antigens and can thereby elicit a rapid and strong response upon restimulation. The adaptive cells are T cells and B cells that express antigen specific receptors, assembled from a high number of genetically encoded elements. This leads to the formation of a broad repertoire of T and B cells with distinct antigen specificity. Although T cells and B cells share the same bone marrow progenitor, they display many differences. T cells become activated upon interaction with antigen-presenting cells (APC) that display antigenic epitopes presented by major histocompatibility complex (MHC)-molecules. The MHC molecules in humans are also called human leukocyte antigens (HLA) since their expression was first characterized on lymphocytes. The HLA class I molecules encompass the classical HLA-A, HLA-B and HLA-C and the non-classical, such as HLA-E and HLA-G. The HLA class II molecules include HLA-DR, HLA-DP and HLA-DQ (Reviewed in [2, 4]). B cells produce antibodies against certain antigens and also act as APC. B cells recognize their antigen in its native form and do not need it to be processed and presented in a specific way, as T cells do.

The innate and adaptive immune systems collaborate. The innate response acts as first-line defense but at the same time contributes to the activation of the adaptive immune response. In this thesis I will focus on the biology of NKT cells, which are cells that act at the interface between the innate and adaptive immune system, and their role in human diseases.

1.2 NKT CELLS

1.2.1 Definition of NKT cells – what's in a name?

NKT cells are a unique subset of lymphocytes that have both immunoregulatory and direct cytotoxic functions. The name NKT cells was first given in 1995 to define a T cell subset in mice that shared the characteristics of NK cells by expressing the NK1.1 receptor [5]. However, already in 1987 several groups had published data on small distinct subsets of T cells in mice expressing a semi-invariant TCR, composed of Vα14-Jα18 co-expressed with either Vβ8.2, Vβ7 or Vβ2 [6, 7]. Later a similar invariant TCR was found in humans composed of the Vα24-Jα18 chain/segment paired with a Vβ11 chain and they were in addition found to express the NK marker CD161 [8, 9]. The NKT cells were found to be highly capable of secreting many different regulatory cytokines rapidly after TCR stimulation [10, 11]. In addition, being strong cytokine producing cells they were suggested to be potent immunoregulatory cells in the immune system [12, 13]. Together these studies indicated the existence of a unique subset of T cells sharing characteristics of both T cells and NK cells, including surface marker expression and functionality. However, now we know that the receptor NK1.1 is not expressed on all NKT cells in the different mouse-strains. Neither is the human NK-receptor CD161 limited to NKT cells, but known to be expressed on other T cell subsets as well [14]. Nevertheless, today we know there are many other NK markers expressed on NKT cells, which will be discussed later on in this thesis. Before going deeper into the NKT cells, I will introduce T cells and NK cells.

1.2.1.1 T cells

The T cell population in our body consists of several subsets with different capacities [2]. CD4+ T cells (T helper cells; Th) are involved in the initiation and regulation of immune responses. CD8+ T cells have an important cytotoxic capacity (cytolytic T lymphocytes; CTLs) and are able to kill cells infected with pathogens, such as viruses and intracellular bacteria. There are also regulatory T cells (Tregs) that are able to regulate immune responses by suppressing lymphocytes via secretion of cytokines [2].

1.2.1.1.1 Maturation of conventional αβ T cells

T cells originate from pluripotent hematopoietic stem cells in the bone marrow, which also give rise to B cells and NK cells. The T cell progenitor migrates from the bone marrow to the thymus. In the thymus the maturation process continues via genetic recombination of TCR genes, leading to surface expression of the TCR. The TCR is a heterodimer of either α-chain and β-chain, or γ-chain and δ-chain. In addition to the highly variable TCR there are other proteins needed to transduce signal upon antigen recognition, the CD3 complex and accessory proteins such as the co-receptors CD4 and CD8. CD3 transduces signals when the TCR binds to antigen-MHC complexes [2, 15].

When conventional αβ T cells progress through the thymus, they differentiate into subpopulations, each with defined repertoires of effector functions. The major subsets are classified by their selective surface expression of CD4 or CD8 [15]. In the thymus, developing T cells first express neither CD4 nor CD8 and then express both CD4 and CD8 (double positive). The double positive T cells will undergo positive selection by

testing the capacity of its TCR to recognize self-antigens presented on MHC molecules on cells in the thymus cortex. The T cells with a TCR recognizing peptides in the context of MHC class II will become CD4+ T cells, whereas T cells with TCRs selected on MHC class I will express CD8. T cells that do not recognize a peptide in the context of a MHC molecule undergo apoptosis (are neglected), since they do not receive the growth signal needed for survival. After positive selection the T cells will undergo negative selection. T cells with a TCR that binds with too high affinity to self-antigens presented in the context of a MHC molecule will undergo apoptosis. Thus, autoreactive T cells are eliminated, which otherwise can cause autoimmune diseases. The negative selection occurs in the thymic medulla before they migrate to the blood stream as mature T cells. Approximately 70% of the mature αβ T cells that finally leave the thymus express CD4, whereas 30% express CD8 [15, 16].

1.2.1.1.2 CD4+ T cells

Activated CD4+ helper T cells are involved in initiating and regulating immune responses by producing various cytokines [17]. CD4+ T cells may express either a Th1- or a Th2 cytokine profile. The Th1-profile involves cytokines such as IFN-γ and IL-2, which will activate CD8+ T cells and macrophages to protect the host from intracellular pathogens. The Th2-profile include cytokines such as IL-4 and IL-13 that will activate and modulate B cell responses and induce them to produce IgE e.g., as well as the activation of eosinophils [17]. Some CD4+ T cells produce the effector cytokine IL-17, but not IFN-γ or IL-4 [2, 18]. IL-17-producing CD4+ T cells (Th17 cells) can induce inflammation and autoimmune diseases, but are also important in the defence against pathogens [19].

1.2.1.1.3 CD8+ T cells

CD8 T cells recognize peptides presented by MHC class I, a molecule that is expressed on all nucleated cells. The activation of a CD8 T cell requires a signal from the TCR through antigen recognition and an additional signal from a co-receptor, signalling survival [20]. During the clonal expansion in an immune response some CD8 T cells will gain effector functions and develop into CTLs. Such effector cells have the capacity to release perforin and granzyme as well as use death receptors, such as tumor necrosis factor related apoptosis-inducer (TRAIL) and FAS ligand (FasL), to kill target cells. They can also secrete cytokines and chemokines (IFN-γ, TNF-α, IL-2, MIP1-α/β) to recruit and activate other cells [20, 21].

1.2.1.1.4 Regulatory T cells

Tregs belong to the CD4+ population but act as immunosuppressive cells controlling the immune responses and autoreactive T cells. They express high levels of the cell surface IL-2 receptor α-chain (CD25), the transcription factor FoxP3 as well as the inhibitory molecule CTLA-4 [22]. Tregs inhibit immune activation by both cell contact-dependent and independent mechanisms and can be reduced in number or functionally impaired in different diseases (reviewed in [22, 23]).

1.2.1.1.5 γδ T cells

Human γδ T cells are unconventional T lymphocytes that account for 1-10% of the T cell population in human peripheral blood [24]. Most γδ T cells are found in the epithelia of the skin and of the small intestines. γδ T cells seem to be important early in

the development of the immune system (reviewed in [25, 26]), and they seem to have a regulatory role during inflammation [25]. The TCR on γδ T cells differs from TCRs on conventional T cells by not recognizing regular peptide-antigens presented on MHC molecules, but instead phosphorylated intermediates of pathogens and also lipid-antigens. Hence, γδ T cells, generally lacking CD4 or CD8, use the TCR more like an innate PRR to recognize its antigen. Upon activation, γδ T cells rapidly produce pro-inflammatory cytokines, such as IFN-γ and TNF-α and they start to express TLRs and react to corresponding ligand [27]. γδ T cells frequently express the activating NK receptor, NK group 2 member D (NKG2D) and interestingly they can kill tumor cells through NKG2D-mediated signalling, independently of their TCR [28].

1.2.1.2 NK cells

NK cells are effector lymphocytes of the innate immune system that are able to kill tumor cells and virus-infected cells [29, 30]. The NK cell was first described in 1975 as a cytotoxic effector cell without a need for prior sensitization [31, 32]. They constitute approximately 10% (5-15%) of the peripheral blood lymphocytes in humans, but are also found in liver, spleen and lymph nodes [33, 34]. Importantly, NK cells also have the capacity to migrate into specific tissue sites when needed [34].

The missing self-hypothesis, describing how NK cells discriminate normal MHC class I expressing cells from virus- and tumor-transformed cells with aberrant MHC class I expression, was postulated more than two decades ago [35, 36]. Today it is known that the balance of signals from an array of activating and inhibitory NK cell receptors (NKRs) control NK cell activity. In contrast to the B- and T cell receptors, the NK cell receptors do not undergo somatic rearrangement. Hence, NK cells can act early and rapidly in the defence against virus and tumor-transformed cells as well as against some intracellular bacterial infections. NK cells are therefore considered as being part of the innate arm of the immune system Importantly, NK cells also play an important role as regulators of immune responses by linking and modifying innate and adaptive immunity [34]. They play a role in some autoimmune diseases, such as type 1 diabetes (T1D) (reviewed in [37, 38]). The role for NK cells in the pathogenesis of T1D is unclear though. Both in mice and humans, NK cells have been suggested to contribute to T1D, by directly or indirectly destroy the β-cells. [38]. It has however also been observed that NK cell numbers are reduced in patients in the early phase of T1D, whereas in the chronic phase the phenotype is changed with reduced expression of activating receptors, such as NKG2D [39].

Human NK cells are defined by their expression of CD56, while lacking the expression of CD3. They can be divided into several functionally distinct subsets based on the expression level of CD56 (Reviewed in [40]). $CD56^{dim}$ NK cells, that also express high levels of CD16, are highly cytotoxic and constitute approximately 90% of the total NK cell population in human peripheral blood [41]. The $CD56^{bright}$ NK cell subset is focused on cytokine production and has an immunoregulatory role [41]. A third NK subset has been described that lacks CD56 expression, but expresses CD16. The $CD56^{neg}$ NK cells are functionally impaired with low cytokine expression and poor cytolytic capacity. Interestingly, some studies have observed an increased frequency of this NK cell subset in chronic viral infections [42-44].

The NK cell activity is under strict control by signals from inhibitory receptors used to sense the presence of classical or non-classical MHC class I molecules on target cells (Table 1). The surface expression of MHC class I molecules may be reduced upon viral or malignant transformation. The first receptor found to negatively regulate and specifically bind to MHC class I antigens, was the Ly49 (expressed by murine NK cells) [45]. In humans the corresponding MHC class I-binding receptors are the killer cell immunoglobulin-like receptors (KIRs) [46]. KIRs, together with the CD94/NKG2A receptor, are the major inhibitory NKRs and recognize HLA-A, -B, –C and -E alleles. However, the KIR family also consists of activating receptors.

Importantly, activation signals from activating NK cell receptor are critical to induce target killing (Table 1). Resting NK cells can only kill target cells upon co-stimulation via several activating receptors simultaneously, whereas cytokine activated NK cells may only need activating signals from one receptor [47]. The receptor NKG2D mediate activation signals upon ligation to MHC class I chain-related proteins (MIC) and UL16 binding proteins (ULBPs) that are up-regulated on the target upon cellular stress [48-50]. The natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 are also important receptors mediating NK cell activation. However, the ligands for the NCRs are not fully characterized today. Moreover, CD94/NKG2C is an activating NKR that binds to HLA-E, as the inhibitory CD94/NKG2A receptor also does. Other activating receptors are 2B4, DNAM-1 and CD2 that bind to CD48, CD155 and leukocyte function-associated antigen-3 (LFA-3) respectively [29]. NK cells also express the Fc receptor CD16 (FcγRIIIa) that can induce antibody-dependent cellular cytotoxicity (ADCC) [30]. Engagement of CD16 mediates a strong activation signal in NK cells and initiates target killing. Not particularly efficient alone, but much better in combination with LFA-1 or 2B4 engagement [51]

Table 1. Examples of human NK cell receptors, their ligands and function *

Receptor	Signalling	Cellular ligand	Function
NKG2D (CD314)	Co-activation	ULBP, MICA, MICB	Surveillance of tumor cells and viral infected cells
2B4 (CD244)	Co-activation	CD48	Interaction with hematopoetic cells
CD94/NKG2A (CD159a)	Inhibition	HLA-E	Sensing MHC class I expression
CD94/NKG2A (CD159a)	Inhibition	HLA-E	Sensing MHC class I expression
DNAM-1 (CD226)	Co-activation	CD112, CD155	Surveillance of tissue integrity
CD2	Co-activation	CD58	Interaction with hematopoetic and endothelial cells
NKp30 (CD337)	Co-activation	B7-H6	NK cell – myeloid cell cross-talk
NKp44 (CD336)	Activation	?	?
NKp46 (CD335)	Co-activation	?	Surveillance of mitotic cells
FcγRIIIa (CD16)	Activation	IgG	Elimination of antibody coated cells (ADCC)
NKR-P1 (CD161)	Inhibition	LLT1	?
Inhibitory KIR family members	Inhibition	HLA class I alleles	Assess loss of MHC class I alleles

*Adapted from Bryceson et al. Immunological Reviews 2006 [29]

1.2.1.3 Invariant NKT cells and other NKT cells

Most NKT cells are αβ T cells with a distinct gene rearrangement of the αβ-segments forming a TCR that do not recognize classical MHC class molecules, but instead glycolipid antigens presented on the non-classical MHC class I molecule CD1d [52]. NKT cells are unique in their response to glycolipid antigens presented by CD1d and are therefore referred to as CD1d-restricted [9, 53]. There are two types of CD1d-restricted NKT cells called type I and type II NKT cells. The type I NKT cells are often referred to as invariant NKT cells since they have a limited diversity of their T cell receptor chains [54]. Invariant NKT cells are known to recognize the marine sponge-derived α-galactosylceramide (α-GalCer) [55, 56]. In contrast, type II NKT cells (also called non-Vα14 and non-Vα24 NKT cells in mice and humans respectively) express a more diverse TCR α-chain repertoire and are not activated by α-GalCer presented by CD1d [57, 58]. The antigens recognized by type II NKT cells are not fully characterized today, but sulfatide and non-lipidic small molecules have been described as ligands [59, 60]. Another characteristic that distinguish type II NKT cells from invariant NKT cells is that they do not express the NK cell marker NK1.1 in mice or CD161 in humans [61, 62]. In addition, there is a small subset of NKT cells with a more diverse TCR repertoire, expressing CD161 (NK1.1) that does not recognize CD1d [13]. These however are generally not referred to as NKT cells. In this thesis, I have focused on CD1d-restricted invariant NKT cells and will refer to them as NKT cells.

1.2.2 The development of NKT cells

The development of NKT cells is thymus-dependent since they are missing in nude mice (lacking the thymus) [63]. NKT cells are derived from the same progenitor as conventional T cells. However, the CD1d restricted TCR of an NKT cell, also generated from a random gene-rearrangement, is composed of the Vα14-Jα18 subunit in conjunction with certain Vβ-genes in mice and Vα24-Jα18 segment paired with a Vβ11 chain in humans [53].

As for conventional T cells, both mouse and human NKT cells develop in the thymic cortex from CD4+CD8+ double positive (DP) T cells [63-65]. However, in contrast to conventional T cells that are positively selected on MHC class I on epithelial cells, NKT cells are positively selected upon ligation of their TCR with a glycolipid presented on CD1d expressed on DP thymocytes ([65, 66] and reviewed in [67]). Importantly, both the glycolipid and the CD1d molecule are required for the positive selection of NKT cells [68]. The homogenic interaction between two DP T cells generates co-signals that are essential for the NKT cell lineage development [69]. The co-signaling pathway is initiated by receptors of the signaling lymphocytic activation molecule (SLAM) family that further engage SLAM-associated protein (SAP) and Src kinase Fyn. These signalling molecules are crucial for NKT cell development ([70, 71] and reviewed in [72]).

NKT cells leave the thymus as immature NKT cells, largely lacking the NK marker NK1.1 in mouse and CD161 in humans, but instead become positive for these markers following maturation in the periphery [53, 63, 73]. At this stage, the NKT cells also

acquire the expression of other NK cell markers, such as NKG2D [55, 63, 74]. NKT cell homeostasis in the periphery does not require ongoing interaction with CD1d, but is highly dependent on IL-15 [75, 76]. Several additional signals, co-stimulatory molecules and transcription factors are needed for the expansion and maturation of NKT cells after the selection in the thymus (reviewed in [53]). A transcription factor that was recently found to be crucial for NKT cell function in mice was PLZF [77]. PLZF-deficient NKT cells do not acquire the same NK cell-like phenotype as wild-type NKT cells, instead they accumulate in the lymph node and display a less activated phenotype with reduced cytokine production.

The exact roles of CD4 and CD8 in the development of NKT cells are not fully understood. The development into either CD4+ or CD4- NKT cells in mice is suggested to occur in the thymus [78], but how is still not known [79]. In humans it is known that there is about 90% over-representation of CD4+ NKT cells in the cord blood and neonatal peripheral blood, but in the periphery only about 40% are CD4+ NKT cells [64, 80]. The earliest precursors detected are CD4+ and CD161^{low} and when the CD4- and CD161+ NKT cells develop is still not known [79]. Whether the CD4- NKT cells develop from CD4+ NKT cells or if they expand in the periphery is still unclear (reviewed in [53]). However, it is suggested that the CD4- NKT cells develop in the periphery with age. Why the CD8 receptor is expressed in some human NKT cells is unclear, but those subsets seem to have distinct functionality [81-83].

1.2.3 NKT cell subsets

1.2.3.1 CD4 expression on NKT cells

NKT cells can be divided into subsets depending on their surface phenotype. There are mainly two subsets of mature NKT cells, either CD4+ and CD4- NKT cells (Table 2). To be more precise, in humans there are CD4+, CD4-CD8+ and CD4-CD8- (DN) NKT cells, while mouse NKT cells are either CD4+ or CD4-CD8- (DN) [79]. Studies have shown that there are functional differences between CD4+ and CD4- NKT cells, even though the differences are not always clear between the subsets. NKT cells are known to secrete both Th1 and Th2 cytokines rapidly upon activation. In humans, CD4+ NKT cells exhibit a mixed Th1/Th2 cytokine production, whereas CD4- NKT cells produce more Th1 cytokines [74, 82, 84]. These differences make them capable of distinct roles in various settings. It has for example been shown that the two subsets have different capacities to protect against tumors *in vivo*, where CD4- NKT cells have a more prominent role as compared to CD4+ NKT cells [85]. Moreover, the CD4-expressing population shows some expression of the IL-2R (CD25), which might suggest that those cells are a fraction of CD4+CD25+ regulatory cells [84]. CD4- NKT cells generally express more of the NK marker CD56, which may be associated with an effector-like phenotype as in CD8 T cells [86]. NKRs such as CD161, 2B4, NKG2A and NKG2D also seem to be expressed to a higher extent on the CD4- NKT cell subset [82, 84]. This could indicate that the CD4- NKT cell subsets have more NK cell-like properties and could initiate rapid cytotoxicity in response to viral infections or tumors.

The two subsets differ also in homing receptors. CD4+ NKT cells are CD62L^{high} and CD11a^{low}, which mean they preferentially home to lymphoid tissues. CD4- NKT cells

are on the contrary are CD11a^{high} and CD62^{low}, which indicates they are distributed in peripheral tissue [87]. The role of the marker CD4 itself is not really understood. Recent publications have shown that CD4 can serve as co-receptor to increase activation [88, 89].

Table 2. Human invariant NKT cell subsets and examples of their receptors and function *

Main subsets	Receptor expressed	Chemokine receptors	Effector function
CD4+	CD161 CD25 CD62L^{high}, CD11a^{low} CD45RO^{+} CD7, CD28	CCR1^{low} CCR2, CCR4, CCR5, CCR7^{low}, CXCR3, CXCR4	Th1/Th2 cytokines (IL-4, IL-13, IL-10 and IFN-γ, IL-2) CD95L (FasL) GM-CSF
CD4-	CD161, NKG2D, 2B4, CD94, CD56, CD57 CD11a^{high}, CD62L^{low} CD45RO^{+} CD7, CD28	CCR1^{high}, CCR2, CCR5, CCR6, CCR7^{low}, CXCR3, CXCR4, CXCR6	Th1 cytokines mostly (IFN-γ, TNF-α) Perforin, GranzymeB

*Adapted from Kim et al. Trends Immunol 2002 **[90]**

1.2.4 The role of NKT cells in the immune system

How NKT cells respond upon an infection and the quality of the response are decided by several factors, such as the lipid antigen presented by CD1d, the APC and its activation status and the presence of inflammatory cytokines [91, 92]. Possible outcomes of NKT cell activation in terms of how other immune cells are affected include: NK cell activation [93], T cell and B cell activation [94, 95] as well as DC-maturation [96]. These events influence down-stream innate and adaptive immune responses in various pathological conditions including some cancer types, autoimmune diseases and infectious diseases [97].

NKT cells comprise about 1% of lymphocytes in mice [14] and 0.1% in humans, but in humans the percentage varies substantially between individuals [80, 98, 99]. In mice, NKT cells are found where conventional T cells are situated, but the percentage expressed in the different tissues varies as well as between different mice-strains. Among the lymphocyte population in mice, the NKT cells are about 30-50% in liver, 20-30% in bone marrow, 3% in spleen, 1-4% in blood, and lung 7% ([14] reviewed in [98, 100]). Because of the very low frequency of NKT cells it is hard to identify them, especially in humans where they are even lower than in mice [98]. The variability in numbers of circulating NKT cell in humans is still unexplained [72]. However, high numbers of NKT cells are more commonly observed in women and a high number of circulating NKT cells is associated with a bias towards an expanded CD4– subset [101]. NKT cells seem to decline with age [102].

NKT cells may have different functions depending on which tissue they reside in. In tumor rejection against a sarcoma cell line in mice, the NKT cells derived from liver had much more potential in killing the tumors than NKT cells from thymus and spleen [85]. Another study indicates that NKT cells from pancreas and liver, but not spleen, are able to inhibit viral replication in mice infected with lymphocytic choriomeningitis virus (LCMV). This difference could be explained by that NKT cells in pancreas and liver express high levels of OX40 and the interaction with OX40L on plasmacytoid DCs (pDCs) seemed to have an antiviral infect by activation of type I IFN production by pDCs [103].

NKT cells can through secondary effects of their cytokine burst activate several other cell types, such as CD4+ Tells, CD8+ T cells, macrophages, NK cells, B cells and recruit myeloid DCs [13, 73]. However, not only cytokines affect other immune cells but also direct cell-cell interaction. For example, direct interactions between marginal zone B cells and NKT cells may be important to increase antibody production, as this effect could be blocked by anti-CD1d and anti-CD40L antibodies [104]. Cross-talk between Tregs and NKT cells occurs as well, where suppression of NKT cells by Tregs is cell-contact dependent and NKT cells can affect Tregs through IL-2 production [105].

1.2.5 Regulation and triggering of NKT cell activity

1.2.5.1 CD1d and its ligands

The CD1 molecules were first identified using monoclonal antibodies that bound to human thymocytes [106]. CD1 molecules are expressed on different APCs and present both foreign and self-lipids. The structure of the CD1-family is similar to the MHC class I heavy chain genes and share the overall domain-formation with MHC class I molecules ([107] and reviewed in [108]). In humans there are five CD1 proteins (CD1a-e) that based on differences in domains can be divided into two groups. Group 1 includes CD1a, b and c, whereas group 2 includes CD1d, while CD1e is an intermediate form, expressed intracellularly [109] (reviewed in [110]). The two groups have different functions. Rodents have only CD1d and they carry two homologous CD1d genes, which are very similar to the human CD1d. CD1 family genes have also been found in sheep and rhesus macaque. In cow there is CD1a, CD1e and several CD1b genes as well as two CD1d pseudogenes [111]. There is a low degree of polymorphism of the CD1 genes between species, especially for CD1d. One reason may be the relatively low variation in the antigens presented, compared to antigenic peptides presented on classical MHC molecules (reviewed in [111]). CD1d is expressed on many cell types including APCs such as DCs, monocytes, macrophages, B cells as well as on CD4+CD8+ thymocytes, hepatocytes and intestinal epithelial cells (reviewed in [112]). The fact that the CD1d molecule is evolutionarily conserved between species supports an important non-redundant role in the immune system [113].

1.2.5.1.1 Endogenous CD1d ligands

NKT cells are known to have some reactivity against “self” via a TCR-dependent autoreactivity that can be inhibited by blockade of CD1d [9]. This is independent of exogenous ligands, and the autoreactivity is caused by recognition of self-antigens

presented on CD1d (reviewed in [114]). Self-lipids could be phospholipids, glycosphingolipids and sulfoglycolipids. CD1d is loaded with self-lipids in the secretory pathway of APCs or in the endosomal or lysosomal compartments during recycling [108]. There is a variety of self-lipids shown to bind to CD1d in humans [115].

Some years ago, a β-linked glycosphingolipid isoglobotrihexosylceramide (iGb3) was shown to be an endogenous ligand both in mice and humans [116]. It has been speculated that this lipid could be responsible for thymic selection of NKT cells, however recent findings demonstrate that mice lacking an enzyme that is necessary for the production of iGb3, are not NKT cell deficient [117]. In addition, humans seem to have low levels of the enzyme synthesizing iGb3, which further supports the notion that iGb3 is not critical for NKT cell development [118, 119]. However, it has been speculated that iGb3 might only be needed in very small quantities to induce autoreactivity, even though iGb3 is a weaker agonist than α-GalCer [120]. Self-lipids can, in combination with cytokines or co-stimulatory molecules, trigger NKT cells and therefore play a significant role in NKT cell activation under different circumstances, such as inflammation, infection and cancer [121].

1.2.5.1.2 Exogenous CD1d-ligands

In 1997 the first ligand for NKT cells was identified, the marine sponge Agelas mauritianus-derived α-GalCer, shown to be able to activate NKT cells in mice [122]. Kirin Pharmaceuticals was the first company to produce the synthetic α-GalCer, because the ligand seemed to have good effects on melanoma in mice. The compound is also known as KRN7000 [55]. One year later, α-GalCer was found to be a target for human CD1d-restricted NKT cells [113].

Today there are several exogenous ligands that are known to activate NKT cells (reviewed in [123]). Some microbial antigens have been identified to be presented by CD1d and they all share a common structure similar to α-GalCer that allows binding to CD1d. This structure is composed of a lipid tail that is bound to a α-linked sugar that binds to the NKT cell [124]. Bacterial ligands differ from endogenous ligands because they are α-linked glycolipids (reviewed in [73]). These microbial lipids are found mostly in the cell wall of Gram-negative and LPS-deficient bacteria. NKT cells have been shown to directly recognize α-linked glycosphingolipid and diacylglycerol, expressed by Borrelia burgdorferi and Sphingomonas [125-127]. Some NKT cells, in both mice and humans, recognize soluble CD1d loaded with mannose from the cell wall of the mycobateria [128]. The biological response by NKT cells in response to these glycolipids is IFN-γ and IL-4 production. LPS-positive bacteria, on the other hand, can indirectly activate NKT cells. For example Salmonella typhimurium activates NKT cells via LPS-activated DCs that present endogenous glycosphingolipids, in combination with IL-12 [125].

A number of synthetic analogs of α-GalCer with different capacities to stimulate NKT cells have also been described ([129], and reviewed in [130]). One such analogue, containing a sphingosine chain, induces a Th2-cytokine bias in NKT cells in mice. This compound shows usefulness in reducing a Th1-biased autoimmune disease [131]. In contrast, these analogues have been shown to enhance a Th1-type response in vivo

[132, 133]. These examples show that there is a possibility to direct NKT cell responses, which hopefully can be used therapeutically in the future. By using soluble plate bound CD1d to activate NKT cells it is known that stimulation only via the invariant TCR on NKT cells is sufficient to activate NKT cells [96].

1.2.5.2 Cytokine triggering of NKT cells

NKT cells express a number of cytokine receptors [121], that can affect them in many ways. They are also capable of rapid cytokine release, compared to naive T cells, due to cytokine transcripts constantly expressed [134].

1.2.5.2.1 IL-12 and IL-18

In addition to direct TCR triggering by exogenous CD1d-presented ligands, NKT cells can also be activated indirectly without TCR recognition of exogenous antigen during microbial infection. NKT cells can be activated either via recognition of endogenous ligands in combination with the co-stimulatory cytokines IL-12 or IL-18 [121, 135], or by IL-12 and IL-18 only, produced by DCs in response to stimulation via TLRs [91, 92, 125]. The latter mode of NKT cell triggering is independent of exogenous and endogenous antigen. NKT cells release IFN-γ in response to IL-12 in combination with IL-18 stimulation, independently of CD1d [121, 136]. NKT cells can express CD40L upon activation, which engage CD40 on DCs, to initiate IL-12 production by the DCs [137].

1.2.5.3 Other receptors

All TLR receptors are known to be expressed on NKT cells, except for TLR8, but they seem to have no direct function and NKT cells do not respond to TLR stimulation directly [138]. However, TLR-stimulated DCs promoted NKT cell activation indirectly, especially through ligands for TLR2/6 and 7/8 [138]. TRAIL has been shown to induce apoptosis on certain tumor cell-lines. TRAIL-induced apoptosis may be related to the expression levels of death-inducing receptors on target cells, such as TRAIL-R1 and -R2. TRAIL has been shown to be up-regulated on NKT cells upon activation and has the possibility to induce apoptosis of human acute myeloid leukemia cells [139].

1.2.5.3.1 Other cytokines

Not only cytokines such as IL-12 and IL-18 can activate NKT cells, but there are several combinations of cytokines effecting NKT cells. I will mention some important cytokines influencing NKT cells. IL-15 is crucial for maintenance of NKT cell homeostasis after development in the thymus, in the periphery, which is not dependent on CD1d [76]. IL-7 is suggested to influence the development of NKT cells. IL-7 and IL-18 seem to have the possibility to make NKT cells produce IL-4, a Th2 switch, after TCR engagement [140, 141]. IL-33, a Th2 cytokine, has been shown to induce IFN-γ-production by NKT cells both in combination with α-GalCer or with IL-12, [142]. IL-2 or IL-12 stimulation of NKT cells induces perforin up-regulation in CD4- NKT cells but not in the CD4+ NKT population [82]. In one study, IL-15 and IL-2 in combination with CD1d-expressing APCs stimulated CD4+ NKT cells to produce the Th2-cytokines IL-5 and IL-13 [143].

1.2.6 Function of NKT cells

1.2.6.1 Cytokine production

As mentioned earlier, CD4+ NKT cells produce both Th1 and Th2 cytokines, whereas CD4- NKT cells have a Th1 profile. IFN-γ, TNF-α, IL-2 are Th1 cytokines promoting cellular immunity and an inflammatory response. IL-4, IL-5, IL-6, IL-10 and IL-13 are cytokines characteristic of the Th2 profile, and these cytokines modulate humoral immunity and suppress the Th1 response. The possibility to rapidly produce both IL-4 and IFN-γ upon stimulation with α-GalCer is very significant and unique for the NKT cells [73]. After *in vivo* antigen stimulation in murine models, NKT cells in the liver produce IL-4 and IFN-γ within two hours [144]. The secretion of cytokines gives NKT cells their regulatory role [84]. An IL-17-producing NKT cell subset was recently defined in mice [145]. IL-17+ NKT cells in mice are CD4-, NK1.1- and present in thymus, liver, lung and spleen as a small subset of total NKT cells [79]. In the presence of LPS or LPS-stimulated DCs, IL-17 production by NKT cells is enhanced [146]. Engagement of the TCR, by cross-linking CD3 and CD28 has also been shown to trigger IL-17 [78]. IL-21 and IL-22 are also produced by some NKT cells, which seem to be the same subset that produces IL-17 [78, 147, 148].

1.2.6.2 Granule exocytosis

NKT cells can, apart from producing cytokines, also be directly cytotoxic, either by release of perforin and granzymes or by inducing apoptosis of target via the Fas/Fas ligand-pathway [93, 122]. Perforin is believed to perforate the cell membrane of the target cell helping additional components, such as granzymes, released from the granules to enter the target cell. When inside the target cell, granzymes induce apoptosis by activation of the caspase system in the intrinsic pathway [21]. Moreover, target killing can also be induced through interactions between death receptors expressed on the target cells, such as Fas (CD95) and its corresponding ligand FasL (CD95L) expressed by NKT cells [149]. FasL is expressed mainly by the CD4+ NKT cell subset, hence this mechanism might be used mainly by this subset [82]. Engagement of Fas by FasL induces apoptosis in target cells [21].

1.3 NKT CELLS IN HUMAN DISEASES

Invading pathogens, e.g. viruses, bacteria, parasites or fungi, cause an infection, which leads to activation of the immune system and inflammation. Even though an infection is the most common cause of inflammation, there are other causes, such as external damages or autoimmune diseases. To delineate the role of NKT cells in infection and inflammation is an important area of research today. Their capacity to rapidly produce both Th1 and Th2 cytokines indicates that they have an immunoregulatory role because they directly and indirectly may affect many other immune cells. By studying mice lacking NKT cells (CD1d and Vα14 knock-out mouse models), it has been possible to investigate several disease models where NKT cells are involved in either initiating or controlling immune responses. NKT cells can boost anti-microbial immunity and tumor rejection, as well as suppress autoimmune disease and promote tolerance. Despite this, NKT cell activity can be bad to the host, as in some allergy and autoimmune conditions (reviewed in [97]). The role of NKT cells in different disease settings is discussed more in detail in the next sections.

1.3.1 NKT cells in infectious diseases

NKT cells have been shown to be involved in the response against viral and bacterial infections. Bacteria can activate NKT cells directly or indirectly. Direct activation occurs via presentation of microbial lipid antigens by CD1d to the invariant TCR. Indirect activation is triggered by IL-12 and IL-18 produced by activated DCs presenting endogenous antigens on their CD1d molecules [91]. NKT cells appear also to be involved in antiviral immune responses through several mechanisms [150] although the viral lipid envelope is strictly host cell-derived. I will mention the role of NKT cells in some microbial and viral infections and will go deeper into the field of HIV.

1.3.1.1 NKT cells in viral infections

In human chronic hepatitis C virus (HCV) infection there have been some conflicting studies concerning the frequency and function of NKT cells in blood and liver [151-153]. CD1d expression is up-regulated on cells in the HCV infected liver, indicating that there may be a role for NKT cells in this disease [154]. Stimulation via CD1d supports activation and cytokine production by NKT cells, which may recruit NK cells and HCV-specific T cells to the site of infection. However, NKT cells in HCV infected subjects have an activated phenotype with increased expression of CD69 and increased production of Th2-cytokines, which could contribute to the pathogenesis and development of hepatic fibrosis ([155, 156] reviewed in [150]). In contrast, herpes simplex virus type 1 (HSV1) infection leads to a reduced cell-surface expression of CD1d on dendritic cells due to viral inhibition of CD1d recycling [157]. Kaposi's sarcoma, a herpesvirus associated cancer frequently observed in people with AIDS, also displays reduced expression of CD1d [158]. Reduced expression of CD1d probably helps the virus escape recognition by activated NKT cells.

1.3.1.2 HIV/AIDS

Acquired immunodeficiency syndrome (AIDS) was first reported in 1981 as outbreaks of opportunistic infections and rare types of tumors, including Pneumocystis carinii pneumonia and Kaposi's sarcoma [159]. Those affected displayed loss of CD4+ T cells and immunosuppression. In February 1983, Luc Montagnier and his group in France identified a human retrovirus as the pathogen causing AIDS [160]. This observation was later confirmed by Robert Gallo and his group in the USA [161]. The virus was later named the human immunodeficiency virus (HIV). By 1984 several research groups had isolated HIV-1 and soon thereafter the closely related HIV-2 was identified in West Africa. Both viruses can cause AIDS although HIV-2 is less pathogenic. HIV-1 is the most common of the two and the virus that has caused the world pandemic. HIV-1 is believed to have originally evolved from the simian immunodeficiency virus (SIV) found in certain primates in Africa, such as the chimpanzee, African green monkey and sooty mangabey. Interestingly, in the latter two, SIV does not generally cause any immunodeficiency [162-164].

1.3.1.2.1 The HIV-1

HIV is a member of the Lentivirus subfamily and like other retroviruses it is an enveloped single stranded RNA virus that transcribes its genomic RNA to double-stranded DNA and incorporates its provirus into the host genome. This process is facilitated by two virally encoded enzymes called reverse transcriptase (RT) and integrase. The HIV genome is composed of two positive strand RNA copies, located in a core of proteins (p24). Matrix proteins surround the core that is kept within a lipid bilayer. The envelope originates from the host cell, where the highly glycosylated HIV-1 envelope glycoproteins, gp120 and gp41 are inserted. The genome contains three structural genes, which are found in all retroviruses. There is env, which codes for the envelope proteins gp120 and gp41, gag, which codes for core- and matrix proteins and pol that encodes the viral enzymes (RT, integrase and protease). The HIV genome also contains six regulatory genes encoding regulatory and accessory proteins, called tat, rev, vpr, vpu, vif and nef. The proteins encoded by these genes facilitate and regulate viral replication [165]. There have been three groups of the HIV-1 virus identified, based on differences in the env-gene. These groups are M, N and O, where M is the most common. Group M has further been divided into nine clades (reviewed in [166]).

1.3.1.2.2 The pathogenesis of HIV-1 and the immune response

HIV-1 infection can be divided into three phases, the acute phase, the chronic asymptomatic phase and last the phase when AIDS develops (reviewed in [167]). During the acute phase, following the exposure of the virus and a short incubation period, the virus undergoes intensive replication leading to high virus titres in the blood and a decreased CD4+ T cell count. After up to 12 weeks of acute phase, the disease turns into a chronic asymptomatic phase where the viremia declines to the viral set point that is accompanied with an initial CD4+ T cell recovery. The anti-viral activity of CD8+ T cells is believed to be important in reaching this steady state [168]. The chronic phase can go on for up to 10 years. However, the immune system will eventually become exhausted and display a decrease in the number of CD4+ T cells. An HIV infected subject will, without treatment, sooner or later reach critical levels of CD4+ T cells (below 200 CD4+ T cells/µl of blood), in combination with a high viral load. At this immune suppressed stage the host has reached the last phase AIDS, where

the immune system fails to control common pathogens as well as some opportunistic infections and tumors, which will finally lead to death [169].

The epidemic is largely maintained by sexual transmission of HIV-1 via the genital mucosa, but transmission also occurs via blood, placenta and gastrointestinal mucosa (reviewed in [170]). It is not entirely clear if the virus crosses the genital mucosal epithelial barrier as a cell-bound or free virus (reviewed in [171]). However, the infection is most likely initiated by a single virus [172]. HIV-1 has the ability to infect immune cells expressing CD4, combined with any of the co-receptors CCR5 or CXCR4. However, the first cells to be infected are probably mucosal CD4+ T cells. Innate cells might enhance the infection by recruiting more susceptible T cells to the site of infection [173]. Dendritic cells might also be important early in infection by transporting virus, bound to DC-specific ICAM3 grabbing non-integrin (DC-SIGN), to lymph nodes for continuous transmission [174, 175].

The host anti-HIV response consists of both humoral (complement and antibodies) and cellular immunity. There is an enhanced B cell proliferation and hypergammaglobulinemia, which is reflecting the generalized immune activation that is an important cause in the pathogenesis (reviewed in [176]). Other markers of immune activation, such as the inflammatory cytokines IL-6 and IL-8, are increased [177]. High T cell turnover [178] and increased expression of the activation marker CD38 and the programmed death-1 receptor (PD-1) are also characteristic of this disease [179, 180]. Immune activation is predictive of overall disease progression [181].

1.3.1.2.3 The challenges in finding a cure for AIDS

Since the discovery of AIDS in 1981, there have been more than 25 million deaths caused by HIV. Today about 33 million people are infected world-wide and there are approximately 7 000 newly infected individuals every day according to a WHO report from 2008 [182]. A lot of discoveries in HIV-related research have been made since the mid 80's, especially within disciplines such as molecular virology, pathogenesis of the disease and epidemiology. The first breakthrough in the treatment of HIV-1 was in 1987 with the discovery of the nucleoside analogue zidovudine (AZT). AZT is a RT inhibitor, which showed especially good reduction in mother to child transmission (from 26% to 8%) [183]. The second breakthrough came with the develpoement of the protease-inhibitors that had the possibility to inhibit more than one viral enzyme. The development of combination therapies that includes at least three antiretroviral drugs, known as highly active antiretroviral therapy (HAART), has been shown to have better effect and reduces the risk of drug resistance [184, 185]. Unfortunately, there are still toxic side effects and despite combination therapy drug resistance is still a problem [166]. The most recent drugs used for HIV-treatment are the fusion- and integrase inhibitors [186]. Just recently, at the 18th international AIDS conference in Vienna, it was announced that treatment with a vaginal microbicide gel containing an RT inhibitor leads to a 38% reduction of HIV transmission [187]. Although, there have been several large HIV vaccine efficacy trials conducted during the last years, there is still no effective vaccine available. The Merck "STEP" trial, with an Adenovirus 5 (Ad5)-vector based HIV-1 vaccine, which aimed to mount a cellular immune response, failed [188]. A recent vaccine trial conducted in Thailand (the RV144 trial) with a combination of two vaccines, used separately previously and then with no effect,

indicated a low level of protection of 31% [189]. Although there is still a long way before we have a vaccine that is able to eliminate the virus, this study has given renewed optimism for the development of a vaccine against HIV.

1.3.1.3 The role of NKT cells in HIV-1 infection

NKT cells are considerably affected by HIV-1, both in number and function, suggesting that NKT cells may play a role in HIV pathogenesis [190, 191]. The CD4+ NKT cells express the major receptor used by HIV to enter the cell and moreover, the co-receptor CCR5 is highly expressed on most NKT cells. CD4+ NKT cells are more easily infected than conventional CD4+ T cells. Interestingly, there is an inverse correlation between high viral load and low CD4+ NKT cells [87, 192, 193]. In addition, in a study by Sandberg et al., it was shown that CD4+ NKT cells were selectively depleted when exposed to HIV-1 in vitro [87].

It is known that HIV-1 preferentially infects effector memory CD4+ T cells, because they express high levels of CCR5 [194]. CCR5 is known to be the co-receptor for most infecting HIV-1 strains, when the virus is transferred to a new host. People having a deletion in the CCR5 gene are mostly resistant to HIV-1 [195]. Viruses using CCR5 as co-receptor are called R5-tropic viruses, whereas the other most common viral strains are called X4-tropic, because those viruses use CXCR4 as co-receptor. NKT cells have a similar phenotype as effector memory CD4+ T cells with an activated phenotype, characterized by high expression of the leukocyte common antigen CD45RO as well as high expression of the chemokine receptors CCR5 and CXCR6 [196]. High expression of CCR5 and CXCR6, and low expression of CCR7, indicate that NKT cells can migrate into tissues and sites of inflammation. Migration to mucosal tissues would make NKT cells having a higher chance to be infected with R5-tropic viruses. Furthermore, the activated phenotype of NKT cells [197] might suggest that they are constantly activated by endogenous ligands, which would make them good targets for HIV-1. In vitro data supporting this come from studies where NKT cells produce more viral particles than conventional T cells following stimulation with α-GalCer [192]. It has also been observed that NKT cells are more susceptible to R5-tropic viruses than to X4-tropic viruses [192]. This may indicate that CD4+ NKT cells could be early targets in HIV infection.

CD4- NKT cells are to some extent also reduced in HIV-1 infection [198]. It is believed that they are resistant to direct infection [199], but there are probably alternative mechanisms explaining why CD4- NKT cells are depleted. Some studies indicate that in vivo stimulation of NKT cells through its TCR or by IL-12 (that could be produced by activated DCs) can result in rapid loss by apoptosis [136]. This suggests that if there is constant activation of NKT cells in HIV-1 infection, apoptosis of these cells could be the outcome [200]. Similar mechanisms could account for the general reduction of NKT cells, including the CD4- subset. Actually one study suggested that the decreased counts of CD4- NKT cells may correlate to markers of immune activation, which could support this hypothesis [198]. Another reason for the loss of NKT cells in the peripheral blood could be that they migrate into tissues [198, 201].

Importantly, HIV can also interfere with NKT cell activation by down-regulating CD1d on infected dendritic cells via Nef- and Vpu-dependent mechanisms [202, 203]. Down-regulation of CD1d is likely a mechanism that the virus has evolved in order to evade recognition by NKT cells.

HAART, which has been shown to have a good effect on the recovery on CD4+ T cells in HIV-1 infected individuals appears not to have the same effect on NKT cells. Different studies observe that NKT cell recovery on HAART is slow at best [198, 204, 205]. There is however one study pointing to an increase in CD4- NKT cells counts, but this phenomenon has not been confirmed in other studies yet [206]. However, Moll et al. observed that antiretroviral treatment (ART) in combination with administration of IL-2 resulted in a significant increase in NKT cell numbers [205]. Administration of IL-2 has previously been shown to increase the number of conventional CD4+ T cells in both chronic and primary HIV-1 infections [207]. The study by Moll et al. was conducted in a cohort with primary infected patients that were either treated with ART or ART in a combination with IL-2. Blood samples were collected at the time when the patients were treatment naïve and during treatment. The expansion of NKT cells following treatment with ART and IL-2 occurred in both the CD4+ and CD4- NKT cell population and the proportion of NKT cells expressing the HIV-1 co-receptor CCR5 decreased over time, possibly making them less susceptible to infection.

1.3.1.4 The role of NK cells in HIV

NK cells can most likely contribute toward limiting HIV-1 replication [208]. There is an expansion of cytolytic $CD56^{dim}$ NK cells during acute infection, which is reduced after the emergence of HIV-specific CD8 T cells [209]. However, during chronic infection there is a skewing of NK cell subsets, with a decrease in the $CD56^{dim}$ cytolytic cells and an expansion of $CD56^{neg}$ NK cells [210, 211]. The increase in these functionally impaired NK cells may contribute to the overall loss of NK cell capacity in HIV-1 infection. There are also changes in expression of NK cell receptors and their ligands in chronic HIV-1, leading to changes in NK cell functionality. For example, the NCRs are down-regulated as well as some of the ligands of the activating receptor NKG2D, leading to a decrease in cytolytic ability [212, 213].

NK cells are an important source of the C-C chemokines, macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES, which suppress HIV-1 entry into cells by competitive binding to the co-receptor CCR5 [214, 215]. An additional protective effect is exerted by the combination of HLA-Bw4 and KIR3DS1 [216]. This HLA-KIR combination is associated with delayed progression to AIDS, increased function of NK cells and a decrease in immune activation [217, 218]. NK cells can be directly cytolytic against infected cells and NKG2D seems to be important for this [219].

HAART decreases the number of the functionally impaired $CD56^{neg}$ NK cells, and restores some of the phenotypic alterations observed in HIV-1 infection [220]. IL-2 therapy in combination with ART in HIV-infected subjects expands NK cells [221], and in particular the cytolytic $CD56^{dim}$ NK cells. With these positive results from treatment so far and the possible benefits of NK cells during HIV-1 infection, more research on these cells is needed [222].

1.3.1.5 NKT cells in non-viral pathogenic infections

As mentioned previously, some microbial lipids expressed in bacterial cell walls are presented by CD1d and recognized by NKT cells [123]. α-linked mono-galactosyl diacylglycerol, a glycolipd from *Borrelia burgdoferi,* stimulates NKT cell proliferation and cytokine production via TCR ligation in both humans and mice [126]. *Sphingomonas* species represent another example of such activation [127]. Both these bacterial infections are worse in NKT cell-deficient mice, indicating that NKT cells have a protective role against these infections. Salmonellosis is an infectious disease where it has been demonstrated that NKT cells have a protective role by production of IFN-γ [125]. The activation of NKT cells is not direct in this disease; instead *Salmonella* bacteria activate DCs to produce IL-12 through activation by LPS binding to TLR4. Hence, NKT cells become activated via stimulation by IL-12 in combination with TCR stimulation by endogenous ligands presented on CD1d on the DCs [91, 125]. In *Brucella* infection, a gram-negative bacteria causing "Malta fever", CD4+ NKT cells have been shown to have a protective role by impairing the intramacrophagic growth of bacteria. In this case it seems to be mainly through up-regulation of FasL and release of cytolytic molecules [223]. There is also increased level of IFN-γ that can activate adaptive immune cells. These mechanisms of activation seem to be dependent on CD1d expression on macrophages. However, the exact mode of activation via CD1d is not yet established, but the protection is increased in the presence of α-GalCer [223]. In *Mycobacterium tuberculosis* (*Mtb*) infection a similar mechanism with intracellular killing of bacteria has been reported in mice pre-treated with α-GalCer [224]. In both mice and humans infected with *Mtb*, there is an alteration in the NKT cell population [225, 226]. A recent *in vivo* study in mice has shown that NKT cells recognize *Mtb*-infected macrophages and produce IFN-γ in a CD1d-dependent way. The NKT cell effector function is dependent on IL-12 and IL-18 produced by the infected macrophages [135].

NKT cells have been studied also in parasitic infections. Using a mouse-model for infection with *Trypanosoma cruzi,* Jα18$^{-/-}$ mice have been shown to have an increased parasitic burden [227]. In Leishmania infection, there have been studies showing an increase in parasite growth in CD1d-deficient mice [228], but also studies indicating that NKT cells do not provide any protection [229]. The role of NKT cells in leishmaniasis seems to be dependent on the mouse strain used and needs to be further investigated (reviewed in [230]).

1.3.2 NKT cells in non-infectious diseases

1.3.2.1 Allergy

NKT cells are required for the induction of airway hyperreactivity (AHR), also called allergic asthma, in murine experimental models. This disease is driven by a Th2-response, with elevated IgE-levels and recruitment of eosinophils. In the absence of IL-4 and IL-13 producing NKT cells, there is no development of AHR [231, 232]. One study focusing on patients with bronchial asthma observed that there is an extensive increase in CD4+ NKT cells dominating the Th2-response [233]. However, these results were questioned, and serious methodological concerns were raised [234]. An increased expression of CCR9 on NKT cells has been observed in humans with AHR,

which could explain the recruitment if NKT cells to the lungs [235]. Furthermore, in a recent study in mice it was observed that CD1d-blockade leads to a reduced frequency of AHR [236]. Results from a mouse model for ozone-induced AHR indicate a role for IL-17 producing NKT cells in the development of the disease. These NKT cells express the IL-17RB, which is the receptor for IL-25 (IL-17E, a member of the IL-17 cytokine family). IL-17RB-expressing NKT cells were detected in the lung and upon IL-25 stimulation produced a lot of IL-13 [237, 238]. In the absence of these NKT cells there was no IL-25 driven AHR.

1.3.2.2 Atopic eczema

Atopic eczema (AE) or atopic dermatitis is a common chronic relapsing inflammatory skin disease, with IgE-mediated sensitization to food or environmental allergens (reviewed in [239]). Atopy is the general medical term for allergic conditions such as high fever, asthma or this type of eczema. The prevalence has more than doubled over the last 30 years and atopy is affecting about 15-30% of children and 2-10% of adults in industrialized countries today. Contributing factors to the disease include genetic predisposition, defect in skin barrier function as well as environmental triggers, such as the gram-positive bacteria Staphylococcus aureus and the commensal yeast Malassezia sympodialis. Approximately 50% of the adult AE patients express IgE specific to M. sympodialis. The yeast M. furfur is present in the normal microflora of human skin, but can act as an allergen in AE patients [240].

In the initiation phase of AE, there is a Th2-like cytokine pattern in the lesions (including IL-4 and IL-13). IgE does not occur in the beginning, but first after a few months suggesting that the skin is the site of sensitization [241]. Epidermal DCs, which are Langerhans cells and inflammatory DCs, both express a receptor for IgE, which is up-regulated in AE [242]. These cells take up and present allergen to T cells. Langerhans cells contribute to Th2 activation when encountering allergen, while inflammatory DCs contribute to a Th1 polarization (reviewed in [239]). The mechanisms for this are not yet established. The Th2 cytokines will stimulate B-cells to produce IgE. The production of the Th1 cytokines IL-12 and IL-18 by inflammatory DCs is characteristic of the chronic stage of AE. For example, elevated levels of IL-18 have been found in the serum from patients with AE. IL-12 and IL-18 switch the response from a Th2 response towards a Th1 response, which is characteristic of the chronic stage of the disease. However, in the absence of IL-12 in mice, IL-18 alone induces IgE production and promotes IL-4 production from T cells [243].

1.3.2.3 The role of NKT cells in atopic eczema

The role of NKT cells in atopic diseases is controversial. Findings reported from mouse and human studies are somewhat contradictory and at present there is no consensus on the role of NKT cells in AE. CD1d is expressed by keratinocytes in the skin and NKT cells have been found at sites of allergic contact dermatitis in mice [244]. NKT cells might further be a big source of IL-4 and could have an important role in the recruitment of IgE [145]. In humans with AE it has been observed that NKT are reduced, in particular the CD4- NKT cell compartment [245, 246]. On the other hand, one group reported that the number of NKT cells was increased in AE patient [247]. To ascertain the exact role of NKT cells in AE, more studies need to be done [245].

1.3.2.4 NKT cells in autoimmune diseases

One of the roles for NKT cells appears to be in the regulation of immune tolerance and control of autoimmunity. In humans it has been observed that NKT cells are reduced in several autoimmune disorders [248]. Altered function or depletion of these cells has been observed in patients with rheumatoid arthritis [249] and multiple sclerosis [250]. Functional defects and reductions in NKT cell numbers have been observed in murine models of autoimmune diabetes [251] and multiple sclerosis (allergic encephalomyelitis) [252]. In humans with T1D, contradictory studies observed either a defect in NKT cell function in patients with T1D [253], no change in NKT cell numbers or function [254] or increased numbers in patients with a recent-onset of the disease [255].

1.3.2.5 NKT cells in cancer/tumor rejection

There are plenty of studies on the role of NKT cells in cancer and tumor immunity (reviewed in [256]). NKT cells are decreased in number and/or functionally impaired in several types of cancer [257, 258]. The first documented effect of NKT cells in tumor immunity was demonstrated when α-GalCer was shown to be an immunotherapeutic agent with the ability to promote NKT cell-dependent rejection of tumors [122, 259, 260]. Furthermore mice lacking NKT cells have an increased tumor incidence as compared to wild-type mice [260, 261]. It has further been observed that it is mainly the ability of NKT cells to produce IFN-γ that is essential for their anti-tumor activity, by recruiting NK cells and cytotoxic T cells [261]. NKT cells also have an important role in protection by activating NK cells, by producing IL-2 or by stimulating DCs to produce IL-12 via CD40/CD40L interaction [93, 137]. In a recent study based on patients with colon cancer, it was reported that there was an increased frequency of CD4+ NKT cells in blood, liver as well as in the tumors. Such a skewing of the NKT repertoire, with less CD4- NKT cells producing high levels of IFN-γ and instead increased levels of Th2 cytokines, can actually inhibit the activity of tumor-antigen specific CD8+ T cells and enhance tumor growth [262]. These data suggest that novel NKT cell-based therapies should try to restore CD4- NKT cells at the tumor site.

A clinical immunotherapy study based on α-GalCer pulsed DCs demonstrated an increased activation of the adaptive immune system caused by NKT cells [263]. Moreover, anergy was not observed using this strategy, as compared to treatment with α-GalCer alone, in mice [263, 264]. Clinical trials with α-GalCer alone have also been conducted [265] as well as transfer studies with autologous NKT cells [266], however none of these clinical studies have so far shown significant results in reducing tumors. New studies in mice are showing that in vivo killing of tumors by NKT cells is dependent on the amount CD1d expressed and the NKT cells are most effective against CD1d-expressing tumors [95, 267].

2 AIMS OF THE THESIS

The overall aim of this thesis was to get a deeper understanding of human NKT cells, their function in general as well as their role in human diseases. We wanted to investigate how the activity of NKT cells is modulated by receptors other than the TCR and by cytokines, and to improve our understanding of these cells in the context of HIV-1 infection. The specific aims were:

1) To determine the functional significance of expression of NKG2D and other NKRs on NKT cells.

2) To investigate the functional capacity and phenotypic characteristics of NKT cells retained in chronically HIV-1 infected subjects.

3) To study the numerical and functional responses of NKT cells and NK cells to IL-2 treatment in patients with primary HIV-1 infection.

4) To determine the capacity of IL-18 to trigger human NKT cell reactivity to endogenous CD1d in the absence of exogenous antigen.

5) To investigate the role of NKT cells in AE.

3 RESULTS AND DISCUSSION

Here I will discuss the results from the papers included in this thesis in three main separate sections. The first section focuses on the modulation of NKT cell function by different receptors, other than their invariant TCR. I will concentrate on NKG2D and PD-1. The second section focuses on how skewing in the NKT cell compartment can occur in diseases and how this can affect the disease outcome. Finally, I will discuss how cytokines can affect the NKT cells and their function, focusing on IL-18 and IL-2. **Paper I-IV** can be read in full at the end of this thesis.

3.1 MODULATION OF NKT CELL FUNCTION BY RECEPTORS OTHER THAN THE TCR

The NKT cell activity may be governed not only by signals from the TCR alone, but can also be modulated by signals such as cytokines and various cell surface receptors. Some NK markers have been found on NKT cells, but the role for these receptors is not yet known and there are also additional receptors not yet studied. The function of NKRs such as NKG2D, 2B4, CD94/NKG2A and KIRs on NKT cells is not yet fully characterized [82, 84]. Several studies have demonstrated that the function of NKT cells may be modulated in different disease settings, and this has been linked to alterations of receptor expression [103, 158, 203]. In paper I of this thesis, we have reported that NKG2D can induce cytotoxicity by human NKT cells independently of the TCR. In paper II of this thesis, we have reported that the inhibitory programmed death-1 (PD-1) receptor (CD279) is up-regulated on human NKT cells that are retained in HIV infected subjects, indicating functional exhaustion.

3.1.1 The activating NKG2D receptor on subsets of NKT cells

In paper I we demonstrate that CD4- NKT cells can degranulate and kill target cells lacking CD1d via signals from the NKG2D receptor, independently of their TCR. To the best of our knowledge, this is the first study demonstrating that NKT cells are able to kill targets via another receptor than the TCR. NKG2D was primarily expressed on the CD4- NKT cells, and NKG2D-mediated killing was accordingly mainly performed by this subset. A similar expression pattern of NKG2D on NKT cells has been observed in other studies [82]. Interestingly, the perforin expression by NKT cells coincides not only to a high extent with the CD4- subset [82], but also with the cell surface expression of NKG2D (figure 2 in paper I). Moreover, our data indicate that both NKG2D and perforin are localized in the immunological synapse following target cell interaction (figure 3 and 4 in paper I). Hence, based on our in vitro data in paper I, we suggest that human NKT cells have the capacity to mediate NK cell-like cytotoxicity triggered by NKG2D in the absence of TCR and CD1d interactions. These results are intriguing since NKG2D has been shown to be important in tumor immuno-surveillance [50, 268], and in the control of cytomegalovirus (CMV) infection [269, 270]. However, further studies are needed to clarify the in vivo role of NKG2D in NKT cells in the context of infection or malignancy.

Resting NK cells, not activated by cytokines, appear to need co-stimulation by a combination of receptors in order to become activated in vitro [47]. A combination between either two of the receptors NKG2D, 2B4, DNAM-1, CD2 and NKp46 triggers NK cells. We performed experiments to investigate if any of the activating receptors 2B4 and DNAM-1, which were also expressed on the NKT cells, were able to induce degranulation and if there was any synergy between NKG2D, DNAM-1 or 2B4. When assessing the functional consequences by reverse ADCC (rADCC), where receptor-specific agonistic monoclonal antibodies can be combined to co-stimulate selected NKRs (as in figure 2 in paper I), we observed almost no degranulation capacity by DNAM-1 or 2B4 either alone or in combination with any of the activating NKRs (data not shown). Interestingly, however, NKT cells seem to be able to kill via NKG2D without the aid of other receptors.

A possible caveat in the results in paper I is that NKG2D-mediated triggering and NK cell-like activity could be speculated to happen because the cells are cultured in medium that includes IL-2. The activating NKRs are known to have a co-stimulatory effect to TCR-mediated triggering, but also independent activity in combination with high concentrations of cytokines [271-274]. For example, in chronic viral hepatitis, NKG2D is up-regulated on a subset of CD8+ T cells and increases the effect of the TCR-mediated activation. However, these cells are generally not activated through a NKG2D-signal alone [271]. In another study, TCR-independent function of NKG2D on CD8+ T cells was demonstrated in vitro in combination with high concentrations of IL-2 [272]. High IL-2 concentrations up-regulated NKG2D expression on CD8+ T cells resulting in TCR-independent cytolysis. In a study of celiac disease, it was observed that NKG2D could mediate CD8+ T cell activation and that this activity contributed to the immunopathogenesis of the disease [273]. In this case NKG2D expression and the cellular reprogramming of CD8 T cells was driven by IL-15 [274]. The observations that IL-2 and IL-15 have similar effects are not that surprising since they share similar receptor complexes [275]. In addition, NKG2D signalling is coupled to the IL-15 receptor signalling pathway [276]. In our experiments, the dose of IL-2 (10 ng/ml) we use should be considered low. In addition, the experiments with NKT cell lines were performed just prior to re-stimulation. At this time, the acutely activating effect of IL-2 should have waned. However, to try to overcome this problem we isolated NKT cells from freshly isolated PBMC, and used these NKT cells directly in a 51Cr-release killing assay (figure 5 in paper I). We were able to detect a killing-capacity against K562 cells though killing was not as high as with cultured NKT cells. The lower killing capacity displayed by freshly isolated NKT cells via NKG2D, could be explained by the lack of prior IL-2 stimulation. However, it is probably more likely due to the low numbers of NKT cells and that they are newly bead-separated. However, seeing killing at all from freshly isolated lymphocytes must be considered as a strong indication that this activity exists also in vivo. Furthermore, in our NKT cell cultures we did not see any increase in the expression level of NKG2D compared to freshly isolated NKT cells, as has been seen for T cells exposed to IL-2 [272, 274].

It would be interesting to investigate whether NKG2D plays the role of a co-stimulatory molecule in the TCR-mediated activation of NKT cells, as previously demonstrated with CD8 T cells and γδ T cells [272, 277]. In addition, a recent study

shows that in patients with rheumatoid arthritis there is an elevated expression of NKG2D on CD4+CD28- T cells, and that NKG2D in this setting indeed acts as a co-stimulatory receptor [278].

The complex formation between NKT cells and target cells expressing ligands for NKG2D, but lacking CD1d-expression, happens within minutes. Experiments using confocal microscopy uncovered polarization of NKG2D at the site of interaction between the cells (figure 3 in paper I), as well as perforin polarization (figure 4 in paper I). Both these accumulations occur independently of specific CD3 polarization. This strengthens the notion that NKG2D can activate NKT cells and induce degranulation of cytolytic molecules. One way to visualize activation and synapse formation between T cells and target cells is to stain for tubulin. Tubulin belongs to a family of proteins that make up microtubules. Microtubules serve as structural components within cells and are involved in many cellular processes including mitosis, cytokinesis and vesicular transport [279]. The microtubules grow from the microtubule-organizing center (MTOC). When a T cell is triggered, the MTOC polarizes towards the immunological synapse and the cytolytic granules move along the microtubules toward the synapse, where granule contents are released [279]. We stained for tubulin (red) and observed polarization in the NKT cells that make contact with K562 cells, but no polarization in the target cell itself (Figure 1). To summarize, rapid tubulin polarization happens in addition to polarization of NKG2D and perforin when NKT cells are co-cultured with K562 target cells, indicating that there is activation of cytolysis via NKG2D on the NKT cells independent of the TCR.

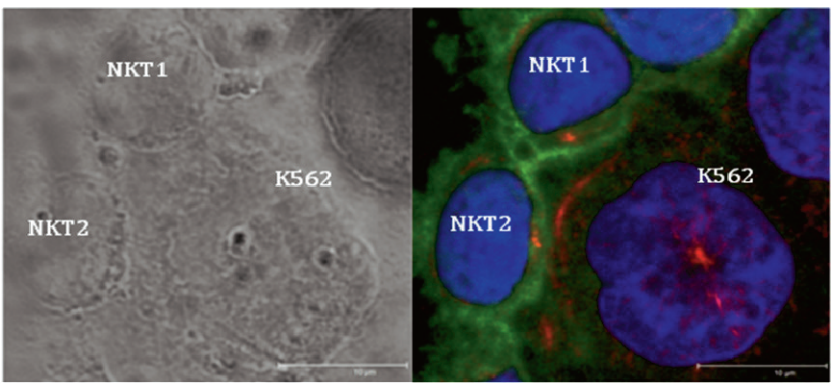

Figure 1. Tubulin polarization in NKT cells upon K562 target cell engagement. Light contrast image and confocal image of NKT1 and NKT2 together with one K562 target cell, after a 15 min co-incubation. CD3+ (green) NKT cells and tubulin (red) in NKT cells and K562 cell. Polarization of tubulin in NKT1 and NKT2 in contact with K562.

Something that could be addressed in future studies is that NKG2D is known to be down-modulated on NK cells following target killing [280, 281]. This phenomenon has also been observed for other cell surface receptors, such as DNAM-1 [282], and could potentially be used to get an indication of involvement of activating NKRs on NKT cells.

A role for NKT cells in immune surveillance against tumors is known, particular from work with TCR-Jα18-knock-out mice, which lack the NKT cell-specific TCR rearrangement and are therefore NKT cell-deficient. Some of these studies confirmed a critical role for NKT cells in protecting mice from spontaneous tumors initiated by the carcinogen methylcholanthrene [260]. The role of the innate NK cell-like NKT cell activity in this effect is however unknown. NKT cells are known to kill CD1d-expressing tumor cells both in vitro [93] and in vivo [267, 283]. In the recent study by Wingender et al., the Ag-specific killing via the TCR was shown to be due to Fas-FasL interaction [283]. Gumperz et al. reported that FasL is mainly expressed on CD4+ NKT cells [82]. Hence, one may speculate that the cytotoxicity displayed by the CD4+ NKT cell subset could to some extent be regulated by the TCR-CD1d interaction and the cell-surface receptor FasL. Still, many tumors do not express CD1d and will therefore likely not be targeted by these NKT cells. In contrast, the CD4- NKT cell population, expressing higher levels of perforin, might kill CD1d-negative targets via innate NK cell-like degranulation regulated by NKG2D (Paper I). Given the recent murine data on the important role of NKG2D in tumor immuno-surveillance, one might speculate that also NKG2D-mediated effector functions in NKT cells may be important [284, 285]. In conclusion, NKG2D-mediated lytic function of NKT cells may be important and contribute to a direct anti-tumor effect in vivo.

As mentioned previously, some viruses such as HIV-1 and HSV-1 down-regulate the CD1d molecule to escape TCR-triggering and NKT cell activation. In such situations, TCR-independent activation via NKG2D may let NKT cells perform anti-viral functions and contribute to viral immunity [157, 203]. However, many viruses have also developed escape mechanisms to inhibit NKG2D-mediated activation of effector cells. HSV-1 down-modulates NKG2D ligand expression [286], and CMV expresses proteins interfering with the expression of several of the ligands for NKG2D [269]. Considering our results, the viral mechanisms to interfere with NKG2D-ligands might have developed to escape not only NK cells, but also NKT cells.

The data on the regulation of NKG2D ligand expression by HIV-1 infected cells are conflicting. Some studies suggest induction of NKG2D ligand expression on infected cells [287, 288], whereas another study indicates reduced expression following infection [213]. The most recent study indicating induction of NKG2D ligand expression (mainly ULBP-2) claims the viral protein Vpr to be responsible for up-regulation. The authors suggest that Vpr might spread to uninfected cells and thereby induce NKG2D ligand expression. Since NKG2D was reported to be the major pathway of NK cell recognition of HIV-1 infected cells [288]. Vpr induced up-regulation of NKG2D ligands may cause increased NK cell-mediated lysis of infected lymphocytes that may contribute to reduced CD4+ cell counts in HIV infected individuals. NKG2D-mediated activation of NK cells may also result in secondary down-modulation of the receptor and impaired NK cell function [280, 281, 288]. This could explain the reduced function of NK cells isolated from HIV patients [208]. Shedding of NKG2D ligands may also lead to impairment of NK cell function, which has been demonstrated for MICA [289]. In fact, one study reports that elevated MICA in serum of HIV infected individuals, likely caused by shedding of MICA from infected CD4+ T cells, correlates with reduced NKG2D expression on NK cells. The NKT cell count in chronically HIV-1 infected individuals is known to be reduced

[290], and the residual NKT cell population has been shown to be functionally impaired (paper II, [291] and Figure 2). Whether the functional impairment is to some extent a secondary consequence of NKG2D-mediated NKT cell activation would be interesting to investigate.

3.1.2 The inhibitory PD-1 receptor on NKT cells

In **paper II** we investigated NKT cells maintained in chronically HIV-1 infected patients and found that they have an immune exhausted phenotype. These NKT cells proliferate poorly in response to stimulation with α-GalCer, have an impaired IFN-γ production and high expression of the inhibitory PD-1 receptor. However, the elevated PD-1 expression was mostly confined to the CD4- subset.

PD-1 was first discovered as an inhibitory receptor on T cells in 1992 [292]. Some years later, the ligands PD-L1 and PD-L2 were discovered. PD-1 transduces inhibitory signals that regulate the balance between T cell activation, tolerance and immunopahology [293]. Today PD-1 is known to negatively regulate many types of immune responses, being expressed on T cells, B cells, NKT cells, activated monocytes and DCs. PD-1 expression on T cells is induced upon activation and is visible within 24 hours. The PD-1 ligands differ in their expression, where PD-L2 expression is more restricted than PD-L1 expression. PD-L1 is expressed in T cells, B cells, DCs, in a wide range of non-hematopoietic cells and is up-regulated on a variety of cells after activation. Both type I and type II interferons (IFNs) up-regulate PD-L1. PD-L2 is inducible on DCs and macrophages and can be up-regulated by IFN-γ. The PD-Ls also are differently expressed between humans and mice and their expression is not yet fully characterized [293].

The PD-1 expression on NKT cells and its function on these cells is not so much studied. NKT cells in mice were shown to constitutively express a low level of PD-1 but upon activation up-regulate the expression [294]. After NKT cells have become activated, they later become hyporesponsive to their ligand [264]. This anergy could be inhibited when blocking the PD-1/PD-L1 interaction and the NKT cells regained their function [294]. In this study, where PD-1/PD-L1 interaction was blocked, together with another study where PD-1 deficient mice were used, the NKT cells regained their capacity to enhance anti-metastatic activities [294, 295]. This could be an important finding when wanting to increase NKT cell activity in the context of some therapeutic approaches. However, the blocking of PD-1-ligand interaction did not prevent anergy when NKT cells were stimulated with Escherichia coli [295]. In our study, we could not regain NKT cell activity by blocking PD-L1 or PD-L2. Hence, inhibiting the PD-1 signal may not in all cases work to regain the function of NKT cells. For example, this treatment would probably not work to support NKT cell activity in chronic HIV-1 infection, as many NKT cells have already been lost. Moreover, the poor function of NKT cells might further indicate that treatment or vaccine regimens including NKT cell activation may not be suitable in this disease.

It was recently found that engagement of the co-stimulatory receptor CD28 on NKT cells releases PD-1 inhibitory signals. Mice lacking CD28 were deficient in cytokine production, both IL-4 and IFN-γ, which could be reversed if PD-1/PD-L1 interaction was blocked [296]. CD28 on NKT cells is known to be important for cytokine production [297], and might be central for NKT cells during development in the thymus [298]. When NKT cells are activated in response to α-GalCer, they rapidly up-regulate PD-1 and the ligands are up-regulated on DCs as well, probably due to IFN-γ [296]. This interaction suppresses cytokine secretion. This recent murine study, however [296], indicates that CD28 signaling can release NKT cells from the inhibitory effects of PD-1/PD-L1 interaction. This suggests the PD-1 receptor in combination with CD28 receptor to be important in the regulation of NKT cell effector functions. In our study and perhaps as well as in the Escherichia coli-study mentioned earlier [295], where PD-1 blocking failed to release the functional impairment (figure 4 in **paper II**), CD28 expression was not investigated. However, since human NKT cells generally do express CD28 [101], perhaps blocking of CD28 interaction with its ligands, would have revealed an effect of blocking PD-1 interaction. It could also be of interest to investigate CD28 expression on NKT cells in HIV-1 infected individuals.

Several reports have found PD-1 expression to be associated with exhaustion of HIV-specific CD8 T cell responses [180, 299]. Furthermore, PD-1 expression was lower in patients with long-term non-progressive disease [299]. It was also shown that blockade of the PD-1/PD-L pathway in vitro led to increased T cell proliferation and effector cytokine production [300]. Therapeutic blockade of PD-1 signalling is being considered as a potential way to enhance immunity in chronic infections. It was recently observed in vivo in a SIV-macaque model that there was enhanced SIV-specific immunity following PD-1 blockade [301]. However, other immune consequences resulting from blocking PD-1 need to be investigated and additional inhibitory receptors associated with CD8+ T cell exhaustion should be addressed to improve the response in chronic HIV infection [302]. However, our results suggest that the poor functionality of NKT cells in this disease is largely independent of PD-1, indicating that such approaches may not benefit the NKT cell compartment.

Our results in **paper II** that the NKT cells remaining in HIV infected individuals are immune exhausted have been confirmed by another study, also showing that the production of Th1-cytokines is impaired. In this study it was observed that the percentage of NKT cells expressing CD161 correlated negatively with IFN-γ and TNF-α production. CD161 is often used to define the maturation state of NKT cell populations, with higher expression reflecting a more mature phenotype [64, 80]. In this study one can hypothesize that CD161 may be a marker of NKT cell exhaustion. However, no blocking experiments have been done to investigate if CD161 may contribute to inhibition of NKT cell function.

3.2 MODULATION OF THE NKT CELL COMPARTMENT IN DISEASE

The NKT cells can be divided into two main subsets, CD4+ and CD4- NKT cells. These subsets have in common that they respond rapidly to antigen stimulation with a high level of IFN-γ production. They do, however, differ in that the CD4+ subset

produce Th2-type cytokines such as IL-4 and IL-13 whereas the CD4- subset does not [82, 84, 101]. How these subsets respond and are affected in different diseases might be important to know since this might affect the outcome of the immune response as well as the disease. For example we know that CD4+ NKT cells are preferentially targeted by HIV-1 infection, and this may have significant clinical importance given the distinct functional profiles exhibited by these two subsets [97]. Here, I will discuss how the NKT cell compartment is affected in HIV and AE (**paper II** and **paper IV**) and speculate how that can affect the development of the disease and the immune response.

In **paper II**, we investigated NKT cells in chronic HIV-1 infection, and discovered a functionally exhausted state of the NKT cell compartment. There have been studies indicating that the CD4+ subset is more sensitive to HIV-1 infection, suggesting that this infection creates a skewed NKT cell population with an overrepresentation of CD4- NKT cells. The result of this could be that the NKT cells remaining in the patients are skewed towards a profile with Th1 inflammatory cytokines. However, our data (figure 4 in **paper II**) suggest that also the function of the CD4- subset may be suppressed in vivo, as the elevated PD-1 expression was mostly confined to the CD4- subset. The functional impairment of NKT cells was broad-based, with decreased cytokine production and proliferative capacity. In chronic HIV-1 infection it is thus likely that the effect on the NKT cell compartment is a broad-based impairment rather than a functional biasing, but that the mechanisms might be distinct between the subset.

NKT cells in HIV infection express an activated phenotype with increased activation markers, which further propose some sort of involvement of these cells [303]. However, what kind of possible anti-viral responses the NKT cells could mediate is at present unclear, but the observation that HIV-1 actively down-regulates CD1d expression in antigen-presenting cells in an evident mode of immune escape supports this idea [203] (Figure 2).

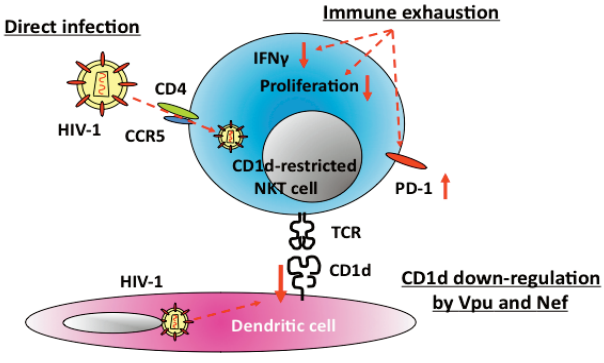

Figure 2. A model illustrating how HIV-1 impacts the NKT cell population.
Based upon three levels. I: Direct infection via the HIV-1 co-receptor CCR5. II: CD1d down-regulation via the HIV-1 regulatory proteins Vpu and Nef. III: Immune exhaustion with impaired IFN-γ production and proliferation capacity as well as up-regulation of PD1 (**paper II**).

In a murine model of asthma it was observed that loss of either PD-L1 or PD-L2 affected the NKT cells and the outcome of the disease in different ways [304]. When PD-L1 was missing, there was an increase in IFN-γ production and reduced AHR. On the contrary, in PD-L2-/- mice there was an increase in IL-4 and a worse AHR-outcome. It is very interesting that these two ligands can have different effects on the function of NKT cells and this needs to be further investigated. In our study we were not able to affect the function of the NKT cells by blocking either of the ligands PD-L1 or PD-L2 (figure 4 in **paper II**). However, this could be very interesting to investigate in other diseases where PD-1 might be up-regulated as a consequence of activation, and at the same time skew the cytokine profile of NKT cells.

IL-18 is a potent pro-inflammatory cytokine. IL-18 alone can stimulate Th2 cytokine production as well as allergic inflammation, but it also enhances IL-12 driven Th1 response (reviewed in [305]). In **paper IV** where we investigated the effect of IL-18 on the activation of NKT cells in AE, the most striking findings were that IL-18 induced CD1d-dependent activation of NKT cells in the absence of exogenous ligands, and that prolonged IL-18 stimulation skewed the NKT cell compartment. In long-term cultures of NKT cells with IL-18 there was a selective suppression of CD4+ NKT cells. In addition, we found that low levels of CD4+ NKT cells were associated with elevated plasma levels of IgE and high levels of IL-18 in the AE patients.

The number of NKT cells varies largely between individuals (0.01-1% of circulating lymphocytes) [55]. However, many different diseases are associated with a reduced proportion of NKT cells in peripheral blood [306]+ref. The decreased pool of CD4+ NKT cells in peripheral blood of AE patients may be part of the pathogenic process or a consequence of the disease. We believe this NKT cell skewing to be IL-18 driven because prolonged exposure of IL-18 in vitro selectively reduces the expansion of CD4+ NKT cells (figure 4 in **paper IV**). Administration of IL-18 alone is sufficient to reduce the splenic NKT cell pool in mice (figure 3 in **paper IV**), which are dominated by the CD4+ subtype. We believe this happens most likely through autoreactive activation of NKT cells since probably only endogenous ligands are present.

Selective reduction of CD4+ NKT cells could thus reduce the anti-inflammatory, Th2-like, effects of NKT cells [54], and thereby add to the chronic Th1 type inflammation described in AE patients [239]. Activation of NKT cells has also been reported to inhibit Th2-type responses, for example by releasing IL-21, which induces apoptosis in IgE+ B cells [307]. We suggest that a skewed NKT cell pool could disturb such inhibitory actions by NKT cells and possibly also contribute to elevated IgE levels in AE patients. This is consistent with the finding that AE patients with elevated IgE levels have a significantly smaller CD4+ NKT cell pool in peripheral blood compared to the patients with total plasma IgE levels within the reference range (<122 kU/L) (figure 5 in paper IV).

The lineage relationships of NKT cells that differ in CD4 expression are not clear; it will be of interest to determine if the production of IL-4 and other Th2 cytokines is limited to terminally differentiated NKT subsets, or alternatively, whether this unique T cell population retains plasticity in its ability to secrete cytokines. To investigate this further, one could culture and expand pure CD4+ and CD4- NKT cells, to observe how

they develop and respond to different cytokines and other stimuli. We have so far succeeded in making these cell-lines and keeping them in culture, as either CD4+ or CD4-. It might be possible to use such NKT cell lines in immune therapy to modulate immune responses. One could speculate that a purified CD4- NKT cell population would produce more IFN-γ and therefore might be good in activating NK cells, which could be used to improve tumor surveillance. Conversely, the CD4+ NKT cells could perhaps be used to treat the autoimmune disease multiple sclerosis, where it has been observed in patients in remission that there is an increase of CD4+ NKT cells and IL- 4 production [308].

What decides which cytokines the NKT cells produce? It could be the type of cytokines they are exposed to, the type of APC, the CD1d-presented ligand or the strength of antigen-mediated TCR stimulation influencing them. As a result of the functional variation of producing pro- and anti-inflammatory cytokines and the importance of this in therapeutic research, it becomes central to determine how NKT cells choose which way to go.

3.3 MODULATION OF NKT CELLS BY CYTOKINES

As discussed above, cytokines can alter the NKT cell repertoire by skewing the proportions of NKT cell subsets. NKT cells express several cytokine receptors [121] that are not only important for the development and maintenance of the NKT cell, but also for triggering of effector functions. However, the cytokine environment is also important for the regulation of NKT cell functions. In this section, I will focus on how cytokines can modulate the NKT cell function. I will discuss how IL-18 affects NKT cells in AE (**paper III**), and how co-administration of IL-2 and ART affects the function and frequency of NKT cells as well as other cells in HIV infected subjects (**paper IV**).

3.3.1 Cytokine-mediated triggering of NKT cells

In **paper IV**, we show that co-culture of human NKT cells and CD1d-transfected cells in IL-18 supplemented medium induce a strong NKT cell response to self-antigens presented on CD1d. We show that IL-18 increases the production of both IFN-γ and IL-4 from NKT cells activated by antigen presented on CD1d. The amount of IFN-γ release by NKT cells following IL-18 stimulation was greater than the release of IL-4 (figure 2 in **paper IV**). A novel finding in paper IV is that IL-18 alone, without the presence of IL-12 or exogenous antigens can activate the production of cytokines from human NKT cells in response to endogenous ligands presented by CD1d, in the context of both DC and CD1d-transfected cells. The long-term effect of IL-18 leads a suppression of the CD4+ NKT cells.

Microbes, such as Malassezia, can induce production of IL-18 by DC and keratinocytes in the initial stage of an AE lesion [240]. Indeed, in patients with AE with an IgE reactivity against Malassezia sympodialis, we observed that the plasma levels of IL-18 correlated with disease severity. Moreover, in the patients with elevated IgE levels

(≥122 kU/L), the plasma level of IL-18 also correlated with total IgE, and this further support that IL-18 may potentially drive the disease in these patients. In HIV-1 infected persons it has also been shown that there is an increased concentration of circulating IL-18, which might affect function of NKT cells and the disease outcome [309].

Studies of the influence of cytokines on NKT cells could help in understanding some diseases, as well as opening the possibility to use cytokines in therapeutic strategies. By using our system for NKT cell culture one could investigate this further. We have studied both IL-7 and IL-15 alone or in combination with IL-2. However, the presence of IL-7 (10 ng/ml) consistently led to less pronounced enrichment of NKT cells, perhaps because the strong anti-apoptotic effects of this cytokine allow survival of higher numbers of other cells as well. We assessed IL-15 together with NKG2D triggering, since the NKG2D signalling is known to be coupled to the IL-15 receptor signalling pathway [276]. However, we did not see any difference regarding NKG2D and its function or a difference in the NKT cell culture when using IL-15.

Based on our data on human NKT cells (**paper IV**) and previously published data demonstrating that IL-18 can increase a Th2 cytokine response by mouse NKT cells having been activated with □-GalCer [140], we propose a model where IL-18 contributes to AE pathogenesis (Figure 3). Elevated and prolonged levels of IL-18 in patients with AE contribute to the pathogenesis by a dual effect, namely firstly by inducing CD1d-dependent activation of NKT cells even in the absence of exogenous ligands and secondly by skewing of the NKT cell repertoire towards an autoreactive phenotype. We speculate that these events may open up for a pro-inflammatory chronic immune response, possibly also including dysregulation of the IgE-production by B cells, and contribute to the subsequent development and maintenance of AE.

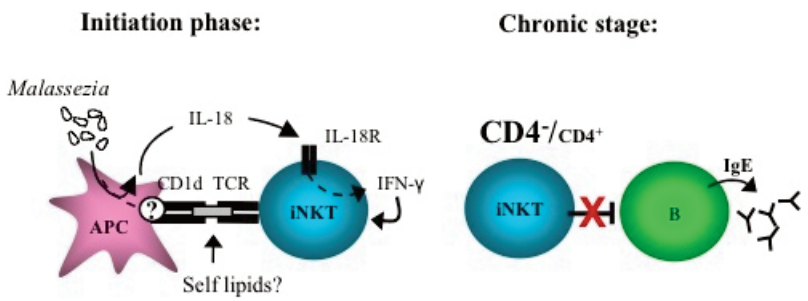

Figure 3. A model showing a possible mechanism for how IL-18 may be a part of the pathogenesis in AE by skewing the NKT cell population and a suggestion for how this can affect IgE production. In the initial phase, high levels of IL-18, produced by DCs following an Malassezia triggering, in combination with recognition of CD1d complexes lead to activation of NKT cells that become pro-inflammatory (release IFN-γ). In the chronic stage of AE, the continuous stimulation via IL-18 induces an IFN-γ driven skewing of the NKT cell repertoire with selective suppression of the $CD4^+$ NKT cells. Hence, these events possibly also lead to dysregulated IgE-production by B cells that contribute to the development and maintenance of AE.

3.3.2 Cytokine treatment of HIV patients

In **paper III** we studied NKT cells, as well as other innate cells, in patients with primary HIV-1 infection initiating ART in combination with IL-2-treatment. The NKT cells responded with a small initial increase in NKT cell numbers, but with no significant functional changes to IL-2 treatment. We found that the NKT cells and NK cells responded with different kinetics and in different ways to IL-2 administration.

In HIV-1 infection there are still many aspects of innate cellular immunity to investigate and understand. The innate immune cells that might encounter the virus early are likely to be important for how other immune cells are activated. NK cells and NKT cells are both rapid responders with the possibility to induce cytokines and also execute cytotoxicity. Combination therapy with ART and cytokines adds an additional complexity to the role of innate immunity in this infection. Previous studies on IL-2 treatment in combination with ART showed an increase in CD4+ T cells, NKT cells and $CD56^{dim}$ NK cells in HIV-1 infected subjects [205, 222]. In this study, we longitudinally followed patients with primary HIV-1 infection initiating ART+IL-2, and simultaneously analyzed the phenotype of NKT cells, NK cells, DCs as well as CD4+ T cells and CD8+ T cells. We aimed to investigate whether there were sustained increases in circulating NKT cell and NK cell numbers one year after stop of IL-2 treatment. In addition we wanted to investigate the effector functions of the NKT and NK cells, and investigate if changes in the two compartments were coordinated.

IL-2 is produced by activated T cells stimulating proliferation of T lymphocytes and NK cells (reviewed in [310]). In NK cells, IL-2 has the additional function of increasing cytotoxic activity and the production of IFN-γ [310, 311]. A functional IL-2 receptor (IL-2R) is expressed on NK cells, and activated T cell subsets. The IL-2R is composed of three subunits and can mediate different signalling depending on receptor composition. In NKT cells it was quite recently shown that IL-2 activation leads to not only proliferation but also the production of both pro- and anti-inflammatory cytokines, IFN-γ and IL-4, respectively [311]. Since IL-2 induces proliferation of many lymphocytes, it has been suggested to be a good complement to other therapies in diseases where there is a need for increased numbers of lymphocytes. Already two decades ago, IL-2 therapy in combination with ART was shown to increase the number of CD4+ T cells in both chronic and primary HIV-1 infection [207]. In treatment of cancer, IL-2 therapy may improve disease outcome [312].

By using multi-color flow cytometry [313], we were able to study phenotypic complexity and functional heterogeneity of several immune cells in parallel. We were able to study CD8+ T cells, CD4+ T cells, NKT cells, NK cells and myeloid DCs (mDCs) phenotypically, plus the function of NKT cells and NK cells. More details of the markers used are found in **paper III**.

To the combined IL-2 treatment the NKT cells responded with a small initial increase in number, and NKT cell responses to α-GalCer antigen were retained but not boosted (figure 1 and 2 in **paper III**). The IL-2 treatment might therefore not durably correct the loss and deficiencies of NKT cells over time (**paper II** and [87, 291]). We looked at expression of different markers, which could indicate exhaustion or activation.

However, there was frequent expression of CCR5, CD161 or PD-1, as expected (paper II and [205, 291]), but there were no changes in response to IL-2+ART treatment. High expression of CD38 is associated with an activated phenotype, and has been associated with HIV-1 disease progression [179]. This marker was slightly decreased during treatment, which could indicate less activation of the NKT cells. However, this was not statistically significant.

In contrast to the NKT cells, the NK cell compartment responded with rapid expansion of the $CD56^{dim}$ effector subset and enhanced IFN-γ production (figure 1 in **paper III**). However, the expansions of both NKT cells and NK cells retracted back towards baseline values 12 months after the end of IL-2 treatment. IFN-γ production in $CD56^{dim}$ subset increased, and as MIP1-β production was unchanged this resulted in a skewing of the functional profile of NK cells that may have implications for NK cells ability to contain HIV-1 (figure 2 in **paper III**). IFN-γ and MIP1-β production by NK cells could be used as indicators of NK cell-mediated response against HIV-1 infection [215].

We also analysed the number of mDCs (defined as CD3-CD19-CD14-CD16-CD56-HLA-DR+CD11c+) and their expression of CD1d. No changes were observed in either of the two parameters (Figure 3), which suggest that DCs are not involved in the expansion of NKT cells observed during treatment.

There is considerable evidence that NKT-DC interaction helps in initiating and regulating immune responses by NKT cells and IL-12 production by DC, directing a Th1 response [137]. In a study investigating patients with advanced melanoma, mDCs were suggested to be one factor contributing to the defect in NKT cell numbers and function due to impaired IL-12 production. It has also been shown that HIV-1 infected DCs fail to produce IL-12 [314]. However, in our study we did not look at changes in mDC function, which should have been interesting to study since that could also possibly affect NKT cell function.

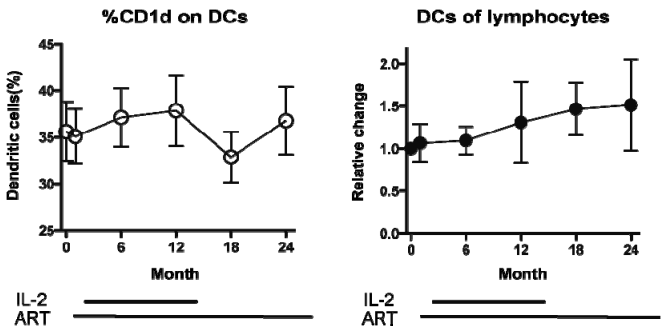

Figure 4. Expression of CD1d on DCs and DC change in number in relation to IL-2+ART treatment

When we were conducting the experiments for **paper III**, two large clinical studies on IL-2 treatment were published [315, 316]. Both these studies compared ART alone or ART+IL-2 and had been going on for about seven years. The end-point of both studies was the occurrence of opportunistic infections or death. The results indicate that even though IL-2 increases the CD4 cell count, the overall outcome was not better in the combinational therapy. The clinical benefits of IL-2 merit further investigation, but clearly these results indicate that IL-2 might not improve the outcome of HIV-1 infection when combined with ART. Our study suggests that IL-2 in combination with ART might be good during the first month to boost especially NK cells, since there is a rapid expansion of $CD56^{dim}$ effector NK cells. Overall, our results help the understanding of innate immune cells in their response to IL-2 treatment in combination with ART.

4 CONCLUDING REMARKS

In this thesis we have reached the following conclusions:

- NKG2D, a receptor for ligands induced by cellular stress, is expressed on CD4- NKT cells. These NKT cells express perforin and can degranulate in response to NKG2D-engagment independently of their invariant TCR.

- NKG2D molecules, perforin and tubulin polarize at the contact site when NKT cells and target cells, expressing NKG2D ligands, form conjugates. This happens independently of CD3.

- NKT cells are able to lyse tumor cells in an NKG2D-dependent manner.

- Although NKT cells are generally lost in HIV-1 infected subjects, some patients retain these cells even in chronic untreated infection.

- NKT cells present in HIV-1 infected patients are functionally impaired with poor capacities to expand and produce IFN-γ in response to stimulation with α-GalCer.

- NKT cells retained in HIV-1 infection display elevated expression of the PD-1 receptor on the CD4- NKT subset; however PD-1 blocking could not restore the functional exhaustion.

- NKT cells respond to ART in combination with IL-2 treatment in chronic HIV-1 infection with a gradual numerical increase, which is not sustained when IL-2 has ended. There appears to be no effect on the IFN-γ and MIP-1β production by NKT cells with neither ART nor IL-2 therapy.

- The $CD56^{dim}$ and $CD56^{neg}$ NK cell subsets respond with a rapid initial increase to IL-2, but this effect is not sustained. However, there is an increase in IFN-γ production by NK cells with IL-2 treatment.

- IL-18 is a potent activator of human NKT cells and promotes a pro-inflammatory CD1d-dependent response, even in the absence of exogenous CD1d-ligands.

- Chronic exposure to IL-18 is inhibitory and skews the NKT cell pool by suppressing CD4+ NKT cells. This pattern is reminiscent of what is seen in AE patients where IL-18 levels are high and CD4+ NKT cells are decreased.

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